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The Localization of Amine Oxidase in the Liver Cell

By JOYCE HAWKINS*
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(*Received* 7 *August* 1951)

Of all the mammalian tissues studied, the liver is the most active source of amine oxidase. This enzyme is known to be associated with the insoluble fraction of the tissue, but no study of its localization in the liver cell has so far been made. Recent work on other enzymes has shown that a number of oxidases are associated with the mitochondria. The enzymes which have been studied include cytochrome oxidase, the succinoxidase system (Hogeboom, Claude & Hotchkiss, 1946), cytochrome reductase (Hogeboom & Schneider, 1950), octanoic acid oxidase (Schneider, 1948), the oxidases of the citric acid cycle (Kennedy & Lehninger, 1949), and the enzymes involved in the synthesis of citrulline (Leuthardt & Müller, 1948). It was therefore of interest to find out whether the mitochondria were also the principal site of amine oxidase activity.

In the present investigation, use has been made of the observation of Hogeboom, Schneider & Pallade (1948), that well defined fractions of liver-cell constituents could be obtained by the differential centrifugation of homogenates prepared in sucrose. In hypertonic sucrose (0·88 M), Hogeboom *et al.* (1948) obtained sedimentation of the bulk of the mitochondria by centrifugation at 24,000 *g* for 20 min., and of the microsomes by centrifugation at 41,000 *g* for 2 hr. Using isotonic sucrose (0·25 M), Schneider (1948) sedimented the mitochondria by centrifugation at 8500 *g* for 10 min., and the microsomes by centrifugation at 18,000 *g* for 60 min.

Using a similar procedure to that described by these authors, various fractions of mammalian liver have been prepared and examined for amine oxidase activity.

METHODS

The preparation of homogenates. Experiments were carried out on rat liver. The liver was minced with scissors, and forced through a wire mesh on to a cooled watch glass. The resulting pulp was weighed and homogenized in a small volume of sucrose solution in an all-glass homogenizer for about 5 min.; more sucrose solution was then added so that 1 g. of tissue was present in 10 ml. of the homogenate. In different experiments the sucrose concentration was either 0·88 M (hypertonic) or 0·25 M (isotonic).

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Centrifugation procedure. About 10 ml. of the homogenate were retained for determination of amine oxidase activity; the rest was centrifuged at 600 *g* for 5 min. to sediment the unbroken cells, incompletely homogenized cell fragments, nuclei, and red blood cells. The sediments obtained were resuspended in sucrose solution up to the original volume. When hypertonic sucrose was used, this preliminary centrifugation of the supernatant was repeated twice more because of the high viscosity of the medium. This will be more fully described in the experimental part.

The supernatant fraction contained the soluble cell constituents and formed elements, mainly mitochondria and microsomes, but a few unsedimented red blood cells and nuclei were also present. This fraction is called the first supernatant, of which about 10 ml. were retained. The remainder was subjected to high-speed centrifugation on an International refrigerated centrifuge, as described in the experimental part. Each of the fractions obtained was made up with sucrose solution so that 1·0 ml. of the fraction corresponded with 1·0 ml. of the homogenate. The temperature was kept as near as possible to 0° throughout.

Manometric procedure. The amine oxidase activity of each fraction was determined in conical Warburg flasks. The main compartment of each flask contained: 1·0 ml. of fraction +0·4 ml. of 0·067 M-sodium phosphate buffer, pH 7·4, +0·2 ml. of 0·1 M-semicarbazide. The side bulb of each flask contained either the substrate, 0·4 ml. of an 0·05 M-solution of tyramine hydrochloride, or 0·4 ml. of water in the blank. The inner compartments contained a filter paper and 0·3 ml. of N-KOH. The determinations were carried out in oxygen at 37°. Readings were taken at 5 min. intervals and the rate of tyramine oxidation was calculated from the oxygen uptake during the first 15 min. of the reaction. Amine oxidase activity is expressed in terms of q_{O_2}, that is, the μl./hr. of O_2 consumed by 1·0 ml. of each fraction (derived from 100 mg. fresh weight of tissue).

Preliminary experiments had shown that the amine oxidase activity of liver homogenates prepared in sucrose was increased by the addition of 0·4 ml. of 0·067 M-sodium phosphate buffer, pH 7·4, per flask. No further increase in activity was observed when more buffer was added.

Nitrogen determinations. It was not convenient to estimate the nitrogen content of the various fractions in the presence of large amounts of sucrose. Nitrogen determinations were therefore carried out on the protein precipitates of each fraction. The protein precipitant used was trichloroacetic acid; 5 ml. of a 2·5% (w/v) solution were added to 1·0 ml. of the fraction. The precipitated protein was transferred quantitatively to micro-Kjeldahl flasks and digested with the sulphuric acid-phosphoric acid mixture described by Campbell & Hanna (1937). Steam distillation was carried out in the Markham apparatus (Markham, 1942). The protein nitrogen content of each fraction is given as mg./ml.

EXPERIMENTAL

Experiments in isotonic sucrose

Four experiments were carried out in which homogenates were prepared in 0·25 M-sucrose. During the first stages the same procedure was followed in all experiments. The enzymic activities and protein nitrogen contents of the different fractions are given in Table 1. In these experiments, the amine oxidase activity of the homogenates varied from a q_{O_2} of 126 to 260, and the protein nitrogen content from 2·38 to 3·35 mg./100 mg. fresh weight of tissue. After low-speed centrifugation at 600 *g*, 71–75 % of the activity of the homogenate was found in the supernatant fraction. The change in the enzymic activity per mg. protein nitrogen was slight.

In each experiment the first supernatant was centrifuged at 22,000 *g* for 45 min. on the refrigerated centrifuge. This was done in order to separate the soluble protein from the formed elements, the mitochondria and the microsomes, which were sedimented. The resulting supernatant had no enzymic activity in two experiments, and 3 and 12 % of the activity of the homogenate in the other two. The protein nitrogen content of this fraction varied from 0·96 to 1·20 mg./ml. Thus centrifugation at 22,000 *g* removed a considerable amount of enzymically inactive protein.

Practically all the enzymic activity of the first supernatant was present in the sediment obtained after high-speed centrifugation. This sediment contained the mitochondria and probably the bulk of the microsomes. The enzymic activity of this fraction per mg. protein nitrogen was higher than that of the starting material. This can be seen from the data given in Table 1.

The further treatment of the sediments obtained in the first high-speed centrifugation differed in the three experiments in which a further centrifugation was carried out. The results are given in Table 2. In Exp. 2 the sediment containing mitochondria and microsomes was suspended in isotonic sucrose, and again centrifuged at 22,000 *g* for 45 min., in order to wash the formed elements and to remove remaining traces of soluble protein. It could be expected that by this treatment the mitochondria and microsomes were again sedimented together. From Tables 1 and 2 it can be seen that after the second high-speed centrifugation the enzymic activity per mg. protein nitrogen of the sediment was slightly increased.

In Exps. 3 and 4 the sedimented mitochondria and microsomes were suspended in isotonic sucrose

Table 1. *Amine oxidase activity and protein nitrogen content of centrifuged homogenates of rat liver in* 0·25 M-*sucrose*

(q_{O_2}: μl. O_2/hr./ml. of fraction. P.N.: mg. protein nitrogen/ml. of fraction. Figures in parentheses give the q_{O_2} and P.N. content as % of that of the homogenates.)

Centrifugation *g*	Centrifugation Time (min.)	Fraction	Exp. 1 q_{O_2}	Exp. 1 P.N.	Exp. 2 q_{O_2}	Exp. 2 P.N.	Exp. 3 q_{O_2}	Exp. 3 P.N.	Exp. 4 q_{O_2}	Exp. 4 P.N.
		Homogenate*	214 (100)	2·38 (100)	260 (100)	2·93 (100)	164 (100)	3·35 (100)	126 (100)	3·04 (100)
600	5	Supernatant*	156 (73)	1·81 (74)	184 (71)	2·28 (78)	120 (73)	2·48 (74)	94 (75)	2·26 (74)
		Sediment	54 (25)	0·75 (31)	90 (34)	0·88 (30)	56 (34)	1·05 (31)	42 (33)	1·19 (29)
22,000	45	Supernatant	0 (0)	0·96 (40)	8 (3)	1·17 (40)	0 (0)	1·15 (35)	16 (12)	1·20 (39)
		Sediment*	151 (70)	0·95 (40)	198 (76)	1·14 (39)	124 (75)	1·34 (40)	100 (79)	1·11 (36)

* Fraction which is subjected to further centrifugation.

Table 2. *Further treatment of the last sediment from experiments shown in Table* 1. *Medium:* 0·25 M-*sucrose*

Exp.	2		3		4	
Centrifugation:						
g	22,000		1,800		8,500	
Time (min.)	45		45		10	
	q_{O_2}	P.N.	q_{O_2}	P.N.	q_{O_2}	P.N.
Supernatant	20 (7)	0·27 (9)	20 (12)	0·58 (17)	20 (16)	0·48 (16)
Sediment	188 (72)	0·88 (30)	92 (56)	0·91 (27)	·62 (49)	0·60 (20)

Table 3. *Enzymic activity and protein nitrogen content of centrifuged homogenates of rat liver. Medium:* 0·88 M-*sucrose*

(Data are given as in Table 1.)

Centrifugation			Exp. 5		Exp. 6	
g	Time (min.)	Fraction	Q_{O_2}	P.N.	Q_{O_2}	P.N.
		Homogenate*	222 (100)	2·65 (100)	180 (100)	2·78 (100)
600	3 × 5	Supernatant*	160 (72)	2·08 (78)	144 (80)	2·17 (78)
		Sediment	49 (22)	1·13 (42)	40 (22)	0·68 (24)
22,000	45	Supernatant	40 (18)	1·39 (52)	24 (13)	1·47 (53)
		Sediment*	108 (48)	0·65 (24)	122 (68)	0·75 (27)
22,000	45	Supernatant	24 (11)	0·22 (8)	20 (11)	0·24 (8)
		Sediment	110 (49)	0·48 (18)	80 (44)	0·41 (14)

* Fraction subjected to further centrifugation.

and centrifuged at a lower speed, at room temperature, in order to separate the mitochondria from the microsomes; the latter can be assumed to have remained suspended in the supernatant, whereas the mitochondria were sedimented. The speed and time of centrifugation were different in the two experiments, as shown on Table 2, but the results were similar. In Exp. 3, the supernatant contained 12%, and the sediment 56% of the activity of the homogenate.

Experiments with hypertonic sucrose

Two experiments were carried out in which the homogenate was prepared in 0·88 M-sucrose. The results are summarized in Table 3. As in the experiments described above, the homogenate was centrifuged at 600 *g* for 5 min., but in order to ensure that all the larger cell fragments had sedimented in the more viscous medium, the centrifugation of the supernatant fluid was repeated twice. The three sediments were pooled and made up to the original volume of the homogenate.

In these experiments the supernatant had 72 and 80% of the enzymic activity of the homogenate, and the activities per mg. protein nitrogen were unchanged. When this fraction was centrifuged at 22,000 *g* for 45 min., a supernatant was obtained which contained the soluble cell constituents and most of the microsomes, and a sediment which contained mainly mitochondria but probably also some microsomes. The sediment was resuspended in 0·88 M-sucrose and again centrifuged at 22,000 *g* for 45 min.

From Table 3 it can be seen that the supernatant fraction containing the soluble material and microsomes had some enzymic activity and that, on resuspension and resedimentation of the mitochondrial fractions, some more enzymic activity appeared in the supernatant. The activity/mg. protein nitrogen of the twice sedimented mitochondria was three times that of the original homogenate.

Separation of mitochondria and microsomes

In the preceding section two different methods have been employed to obtain a preparation of mitochondria free from microsomes. In the first, in isotonic sucrose, the mitochondria and microsomes were first brought down together, and then separated by resuspension and sedimentation of the mitochondria by centrifugation at lower speed (Exps. 3 and 4). In the second method, a fraction of mitochondria was obtained by repeated centrifugation at 22,000 *g* in hypertonic sucrose (Exps. 5 and 6). All the fractions which contained mitochondria stained characteristically with Janus green B at a concentration of 1 in 10,000.

Tables 4 and 5 give a summary of the results obtained. In these two tables the enzymic activities and protein nitrogen contents of the fractions are expressed as a percentage of the values for the first supernatant. Table 4 shows clearly that the removal of soluble cell protein did not noticeably affect enzymic activity. The data given in Table 5 are explained by the removal of some enzymic activity with the microsome fraction. In the two experiments carried out in hypertonic sucrose (Exps. 5 and 6), the enzymic activity per mg. protein nitrogen

was slightly higher than in the two preceding experiments in isotonic sucrose. Whether this was due to any difference in the composition of the mitochondrial fraction, cannot be decided.

Table 4. *Enzymic activity and protein nitrogen content of once sedimented mitochondria plus microsomes*

(Data are given as percentage of the q_{O_2} and P.N. of the supernatant from low-speed centrifugation of the original homogenate.)

Exp.	q_{O_2} (%)	P.N. (%)
1	96	52
2	108	50
3	103	54
4	106	49
Mean	**103**	**51**

Table 5. *Enzymic activity and protein nitrogen content of fractions containing twice sedimented mitochondria*

(Data are given as in Table 4.)

Exp.	q_{O_2} (%)	P.N. (%)
3	77	37
4	66	27
5	69	23
6	56	19
Mean	**67**	**26·5**

DISCUSSION

It has been shown that of the total amine oxidase activity of a liver homogenate, about 50% is associated with those granules which are of the size and staining properties characteristic of mitochondria. It seems likely, however, that the percentage activity resident in the mitochondria is greater than this figure. In the preliminary low-speed centrifugation of the homogenate, about 25% of the total enzymic activity was brought down in the sediment. It can be safely assumed that most of this fraction consisted of unbroken cells and incompletely homogenized cell fragments, which retained enzymic activity. The activity of the mitochondrial fraction expressed as a percentage of the activity of the first supernatant, gives probably a better measure of the contribution of the mitochondria to the amine oxidase activity of the liver as a whole. Table 5 shows that 56–77% of the enzymic activity of the first supernatant was present in this fraction. The finding that amine oxidase is present in the mitochondria is in agreement with a statement by Cotzias & Dole (1951); no full description of this observation has been published.

In isotonic sucrose, the mitochondria and microsomes were brought down together by high-speed centrifugation. This fraction contained practically all the enzymic activity of the first supernatant. It remained constant on resuspension and resedimentation at 22,000 *g*, but was reduced by low-speed centrifugation at 1800 *g*. This indicates that the microsomes also contain amine oxidase, and that together with the mitochondria they account for all the amine oxidase activity of the liver tissue.

It is still under discussion to what extent the microsomes are pre-formed elements of the liver cell. The recent observations of Harman (1950) and of Still & Kaplan (1950), that prolonged homogenization in a Waring blender results in the transfer of succinoxidase from the fraction containing mitochondria to that containing microsomes, raises the possibility that in homogenates prepared by the milder methods used in this paper, there may be some disintegration of the mitochondria. It can therefore not be decided if the amine oxidase activity of the smaller particles is due to the presence of fragmented mitochondria.

The sedimentation of the cytoplasmic granules results in a purification of amine oxidase; this is explained by the removal of a considerable amount of soluble protein which is without activity. From the data given it is apparent that the isolated mitochondria are two to three times more active than the original homogenate.

The particulate matter of the liver cell is known to contain the respiratory enzymes, which have been studied by methods similar to those employed in this work. It is of interest that amine oxidase has a similar distribution, although it is an oxidase for which any connexion with the cytochrome-cytochrome oxidase system has never been demonstrated.

SUMMARY

1. The distribution of amine oxidase in homogenates of rat liver, prepared in either isotonic or hypertonic sucrose, has been studied.

2. Evidence has been obtained that about two-thirds of the enzymic activity is present in the mitochondria; the remainder is present in the microsome fraction.

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The Branched-Chain Fatty Acids of Ox Fat

I. THE ISOLATION FROM OX SUET OF A C_{17} BRANCHED-CHAIN SATURATED FATTY ACID

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The occurrence of high molecular weight branched-chain saturated fatty acids has been reported in certain bacterial lipids (Anderson, 1927, 1941; Velick, 1944; Hofmann & Lucas, 1950) and in wool wax (Weitkamp, 1945). Recent investigations in this laboratory have revealed the presence in butter-fat of (*a*) two isomeric C_{17} saturated fatty acids, each with a methyl side chain (Hansen & Shorland, 1951), (*b*) a multi-branched C_{20} fatty acid (Hansen & Shorland, 1952), (*c*) a C_{18} methyl branched-chain fatty acid (Hansen, Shorland & Cooke, 1951).

In this paper is described a C_{17} branched-chain acid isolated from ox suet by the processes of hydrogenation, fractional distillation *in vacuo*, and fractional crystallization.

EXPERIMENTAL

The fat used in this work was extracted from the suet (15·5 lb.) of a 4-year-old 'prime' heifer (dressed weight approximately 600 lb.). Following the determination of the fatty acid composition of a sample of the fat, 162 g. of the fractionated methyl esters of the C_{18} unsaturated acids were hydrogenated at 150° and at atmospheric pressure, using a nickel catalyst supported on kieselguhr.

Repeated crystallization from 15 vol. acetone at −30° removed the solid products of hydrogenation and yielded

Table 1. *Fractionation of ox-fat liquid methyl esters in micro column*

(Pressure approx. 0·05 mm. Hg.)

Fraction	Wt. (g.)	b.p. (°)	Saponification equiv.	Iodine value (Wijs)	
FL1	0·20	117–122	290·7	3·4	Liquid
FL2	0·60	122–128	290·5	1·6	Liquid
FL3	0·93	128–130	300·7	1·2	Liquid
FL4	0·46	130–131	319·3	2·2	Liquid
FLR	0·44	—	—	—	Viscous liquid

Table 2. *Fractional crystallization of* FL2 *acids*

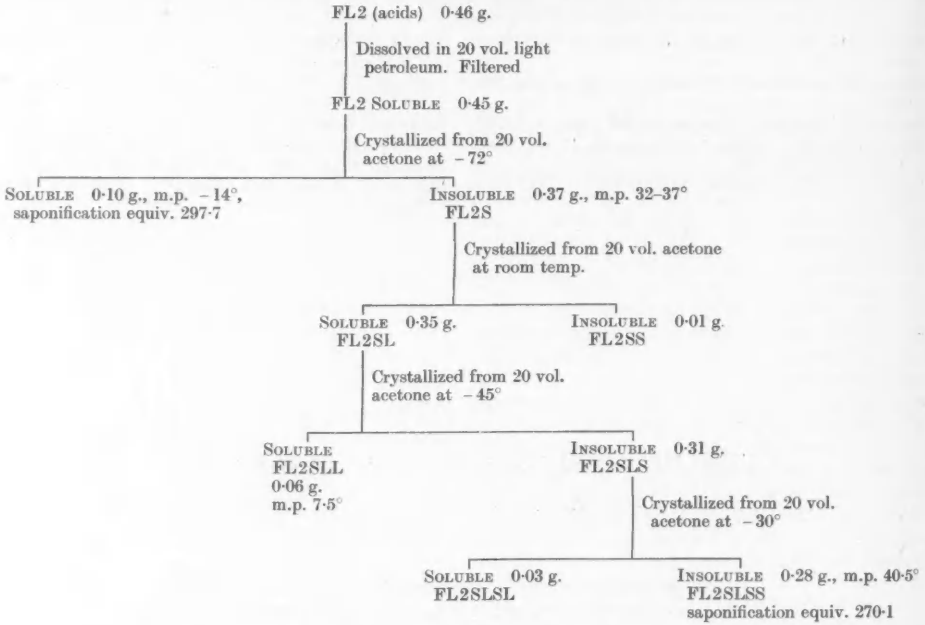

Table 3. *Properties of the* C_{17} *branched-chain fatty acid fraction FL2SLSS*

Fraction	Wt. (g.)	m.p. (°)	Saponification equiv.	Iodine* value (Wijs)	X-ray long spacing (A.)	*C*-Me (%)	Combustion analysis† (%)
FL2SLSS	0·28	40·5	270·1	3·3	33·5	7·0	C 75·5 H 12·5

* $E^{1\%}_{1\,cm.}$ 268 mμ. = 2·2; $E^{1\%}_{1\,cm.}$ 234 mμ. = 5·7.
† Analysis by Dr G. Weiler and Dr F. B. Strauss, Oxford.

6·3 g. (iodine value 2·7) of acetone-soluble 'liquids'. Further separation of 'solid' material was effected by two crystallizations from 10 vol. acetone at −30°, providing 2·7 g. of acetone-soluble 'liquids' (FL) which were fractionated in a micro column. Fractionation data are shown in Table 1.

Fractional crystallization of FL2 acids at varying temperatures, as illustrated in Table 2, resulted in the isolation of fraction FL2SLSS.

X-ray diffraction measurements were made on fraction FL2SLSS, a Philips Geiger X-ray spectrometer being used. Samples were melted on a glass slide and quickly cooled.

The chemical and physical characteristics of fraction FL2SLSS are recorded in Table 3.

DISCUSSION

The results of combustion analysis of the fraction reported in this paper agree closely with the theoretical values required by the formula $C_{17}H_{34}O_2$, namely, C, 75·5, H, 12·7 %. Further confirmation of the molecular weight of the substance is provided by the saponification equivalent of 270·1 which corresponds, within experimental error, with the theoretical value of 270·4 for a C_{17} saturated fatty acid. Evidence for the presence of a side chain is afforded by the *C*-methyl value, which is equivalent to the liberation of 1·26 mol. of acetic acid (cf. Ginger, 1944). Comparison of X-ray diffraction measurements of straight-chain and branched-chain fatty acids distinguishes fraction FL2SLSS, with long spacing 33·5A. (experimental error ± 0·5A.), from normal heptadecanoic acid which has a long spacing of 38·6A. (Francis, Piper & Malkin, 1930.) The long spacing of FL2SLSS does, however, conform with the value of 33·35A. (Arosenius *et al.* 1949) and 33·4A. (Velick, 1947), as reported for C_{17} *iso* and *ante-iso* acids, respectively. The chemical and physical characteristics of the branched-chain acid reported here are almost identical with those of a C_{17} fraction

EuS (saponification equiv. 270·1, m.p. 39·8°, *C*-methyl 8·06%) recently isolated from butterfat (Hansen & Shorland, 1951). A melting-point determination made on an equimolecular mixture of fractions FL2SLSS from ox suet (m.p. 40·5°) and EuS from butterfat (m.p. 39·8°) gave a value of 40·0°. This evidence is consistent with the two fractions being identical.

In order to confirm the purity of this fraction and to identify conclusively other branched-chain acids whose presence has been indicated by examination of the fractions described in Table 1, further quantities of acetone-soluble 'liquids' from ox fat are in the process of investigation.

Although a comprehensive search for branched-chain acids has not been made on the total fatty acid constituents of ox fat, this investigation indicates that their presence in the C_{18} unsaturated acid fraction alone is of the order of 1% of the total fatty acids.

SUMMARY

A C_{17} methyl branched-chain saturated fatty acid isomeric with *n*-heptadecanoic acid has been shown to be present in the fat extracted from ox suet.

We are much indebted to Mr M. Fieldes of the Soil Bureau, Department of Scientific and Industrial Research, Wellington, for X-ray diffraction analysis.

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The Fate of Certain Organic Acids and Amides in the Rabbit

13. CHLORO- AND FLUORO-BENZOIC ACIDS AND AMIDES

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The primary object of this investigation was to determine the effect of chloro- and fluoro-groups upon the stability *in vivo* of the carbamyl group as it occurs in benzamide. When it was found that all the chloro- and fluoro-benzamides were readily converted into the corresponding acids by the rabbit, there was the added interest of comparing the proportion of the dose excreted conjugated with glucuronic acid when an amide was administered, with that excreted when the corresponding acid was given. It had been noted previously (Bray, Neale & Thorpe, 1946) that a greater proportion of a dose was excreted as benzoylglucuronide when benzoic acid itself was given, than when benzamide was administered. (Benzamide was readily hydrolysed *in vivo* to benzoic acid.) This was subsequently explained as a result of a study of the kinetics of this reaction (Bray, Thorpe & White, 1951).

The metabolism of chloro- or fluoro-benzamides does not appear to have been studied previously, but some investigations of the fate of the acids have been reported. Graebe & Schultzen (1867) isolated a compound which was probably the calcium salt of *m*-chlorohippuric acid from human *m*-chlorobenzoic acid urine. Novello, Miriam & Sherwin (1926) failed to detect glycine conjugation of the chlorobenzoic acids in the rabbit, and found that only the para isomer was excreted as a hippuric acid derivative by the dog. Schübel & Manger (1929) observed that 10–70% of doses of *p*-chlorobenzoic acid were excreted unchanged by the rabbit. Quick (1932) found that all three isomers were conjugated with both glycine and glucuronic acid in the dog, glycine conjugation occurring to a much smaller extent with the ortho isomer than with the others; the extent of glucuronic acid conjugation was similar for all three. Only *o*-chlorobenzoic acid was excreted unconjugated to an appreciable extent. Hildebrandt (1903) had also found that the rabbit excreted this acid unchanged, and investigated the formation of chlorobenzoic and hippuric acids from chlorotoluenes. *p*-Chlorohippuric acid was isolated as a

metabolite of *p*-chlorotoluene in the dog. Although pure glycine conjugates were not isolated from the urine after administration of the other isomers, products were obtained which gave *o*- and *m*-chlorobenzoic acids on hydrolysis. In the rabbit, only chlorobenzoic acids were isolated as metabolites of the chlorotoluenes, no glycine conjugation being detected. As far as we are aware, the only recorded account of an investigation of the metabolism of fluorobenzoic acids is that of Coppola (1883), who showed that the dog conjugated all three isomers with glycine.

In the present investigation, the nature of the chief metabolites of the fluoro- and chloro-benzoic acids and their amides has been established by observing the effect of their administration upon the excretion of ether-soluble acid, ester glucuronide, glycine conjugate and ethereal sulphate by rabbits. A new method for the determination of hippuric acids in urine has been worked out.

MATERIALS AND METHODS

Materials. The chlorobenzoic acids were purchased (British Drug Houses Ltd.; Hopkin & Williams Ltd.). The chlorobenzamides were prepared from the corresponding acids via the acid chlorides. *o*- and *p*-Fluorobenzoic acids were prepared from the corresponding ethyl or methyl aminobenzoates by the diazotization fluorination method (Schiemann & Winkelmüller, 1933; Dippy & Williams, 1934). *m*-Fluorobenzoic acid was prepared by the same fluorination method from *m*-aminobenzoic acid. The fluorobenzamides were prepared from the acids via the acid chlorides.

Diet and dosage. The rabbits used were does of 2–3 kg. which were maintained throughout on the diet of pellets and water, previously described (Bray, Ryman & Thorpe, 1947). The compounds under investigation were administered by stomach tube, the acids as solutions of their sodium salts, and the amides as suspensions in water. All the twelve compounds appeared to be non-toxic for the doses (1 g. throughout) used.

Estimation of metabolites. Ether-soluble acid, ester glucuronide and ethereal sulphate were estimated in the experimental urines by methods already described (Bray, Thorpe & Wood, 1949*a*).

Estimation of hippuric acids in urine. Owing to the laborious nature of the method used previously (Bray *et al.* 1946; Bray *et al.* 1947), a method more suitable for routine estimation of many samples has been devised. This is based upon liberation of glycine from the hippuric acids by hydrolysis, and its subsequent colorimetric estimation by a ninhydrin reaction (cf. Milton & Waters, 1949). The urine (25 ml.) is acidified with H_2SO_4 (2 ml., 4 N) and continuously extracted with ether for 6 hr. After evaporation of ether from the extract, the solid residue is hydrolysed by boiling under reflux with HCl (10 ml., 6 N) for 1·5 hr. After cooling, the hydrolysate is adjusted to approximately pH 6·8 by addition of K_2CO_3 (70% w/v), using bromothymol blue (one drop) as indicator. Glycine is estimated in this solution diluted so as to contain between 1 and 7 mg. glycine/100 ml.

Estimation of glycine

The following reagents are used:

Buffer pH 7·0 *containing glycine.* KH_2PO_4 (3·62 g.) and $Na_2HPO_4.2H_2O$ (7·11 g.) in water (1 l.). Glycine (50 mg./l.) added when required for use. The solution is not stable overnight.

Ninhydrin solution. 1% (w/v) in water.

Glycine standard. 90 mg./l. in water, made up immediately before use.

The dilute solution (2 ml.) from the hydrolysis (above) is mixed with the buffer (2 ml.) and ninhydrin solution (1 ml.) in a Pyrex test tube. Tube and contents are heated in a boiling-water bath for 1 hr. using a 'cold finger' to reduce evaporation. After cooling, the purple solution is made up to 25 ml. and the colour intensity estimated in a Spekker photoabsorptiometer using Chance green filter OG 1. A calibration curve was constructed using the glycine standard, appropriately diluted and heated, with ninhydrin alongside the solutions in which glycine was being estimated. The curve is linear over the range 0–180 μg./2 ml., an extinction (drum reading) of 0·1 corresponding to about 22 μg. Control experiments using hippuric acid in water gave recoveries of 99%, S.D. $\pm$3%. Similar recoveries were obtained with substituted hippuric acids. In experiments in which hippuric acid was added to the urine of normal rabbits the average recovery was 97%, S.D. $\pm$6·7.

RESULTS

Excretion of normal metabolites

The average daily excretion of ether-soluble acid (expressed as hippuric acid) by the rabbits used in this investigation ranged from 630 to 688 mg. The average percentage by which individual daily values differed from the corresponding weekly averages used as baselines, for purposes of calculation, was $\pm$11. The corresponding values for reducing material (expressed as glucuronic acid) were 204–228 mg. and $\pm$7%, for ethereal sulphate 38–42 mg. SO_3 and $\pm$13% (5 mg. SO_3), and for conjugated glycine (expressed as hippuric acid) 206–309 mg. and $\pm$11%. Of the material in the ether-soluble acid which was responsible for the ninhydrin reaction, 7–8% was present in unconjugated form, i.e. it reacted without preliminary hydrolysis.

Excretion products of chloro- and fluoro-benzoic acids and amides

Table 1 shows the increases in excretion of ether-soluble acid, ester glucuronide and ethereal sulphate observed after administration of the chloro- and fluoro-benzoic acids and amides. All the compounds administered were excreted mainly as ether-soluble acid, which consisted of a mixture of the chloro- or fluoro-benzoic acid with its glycine and glucuronic acid conjugates. For the amides, the differences between the totals accounted for and the dose administered are probably not due, to any appreciable extent, to the excretion of unchanged amides.

Table 1. *Excretion of ether-soluble acid, ester glucuronide and ethereal sulphate after administration of chloro- and fluoro-benzoic acids and amides to rabbits*

(Dose 1 g. Ranges given in parentheses and number of experiments indicated by superior figures.)

Compound	Average percentage of dose excreted as: Ether-soluble* acid	Ester glucuronide	Ethereal sulphate
o-Chlorobenzoic acid	87 (69–96)[5]	7 (0–11)[5]	0[2]
o-Chlorobenzamide	71 (65–75)[5]	11 (8–18)[5]	0[2]
m-Chlorobenzoic acid	100 (87–109)[4]	23 (12–31)[4]	0[2]
m-Chlorobenzamide	70 (56–89)[5]	1 (0–4)[5]	0[2]
p-Chlorobenzoic acid	69 (65–83)[5]	16 (3–33)[5]	0[2]
p-Chlorobenzamide	84 (58–108)[2]	5 (0–9)[2]	0[2]
o-Fluorobenzoic acid	93 (87–97)[4]	7 (2–10)[4]	0[2]
o-Fluorobenzamide	76 (61–89)[4]	3 (0–9)[5]	2 (0–3)[3]
m-Fluorobenzoic acid	95 (92–97)[4]	10 (5–12)[4]	0[2]
m-Fluorobenzamide	76 (70–82)[4]	4 (2–6)[4]	0[2]
p-Fluorobenzoic acid	85 (72–99)[5]	11 (7–14)[5]	2 (0–5)[2]
p-Fluorobenzamide	79 (70–96)[4]	3 (0–7)[4]	0[2]

* Substituted benzoic and hippuric acids + substituted benzoylglucuronides.

Table 2. *Excretion of glycine conjugates and unconjugated acid and the ratio of the former to the glucuronide excreted after administration of chloro- and fluoro-benzoic acids and amides to rabbits*

(Dose 1 g. Two experiments for each compound. Values for each experiment given in parentheses after average value.)

Compound	Average percentage of dose excreted as: Ether-soluble acid	Ester glucuronide	Glycine conjugate	Unconjugated acid*	*R*†
o-Chlorobenzoic acid	84 (76, 92)	19 (19, 18)	5 (4, 6)	60	0·3
o-Chlorobenzamide	95 (86, 103)	14 (14, 14)	17 (16, 18)	64	1·2
m-Chlorobenzoic acid	75 (74, 76)	23 (22, 23)	51 (46, 56)	1	2·2
m-Chlorobenzamide	94 (87, 101)	17 (17, 17)	74 (73, 75)	3	4·4
p-Chlorobenzoic acid	91 (81, 100)	21 (17, 25)	63 (67, 59)	7	3·0
p-Chlorobenzamide	95 (69, 120)	4 (0, 7)	40 (31, 49)	51	10·0
o-Fluorobenzoic acid	86 (74, 102)	9 (0, 18)	43 (45, 41)	34	4·8
o-Fluorobenzamide	83 (61, 104)	6 (5, 6)	60 (62, 57)	17	10·0
m-Fluorobenzoic acid‡	99 (98, 100)	6 (2, 10)	24 (26, 22)	69	4·0
m-Fluorobenzamide	93 (85, 101)	9 (6, 12)	74 (62, 85)	10	8·2
p-Fluorobenzoic acid	87 (95, 79)	9 (8, 9)	43 (38, 48)	35	4·8
p-Fluorobenzamide	103 (85, 121)	6 (5, 6)	93 (88, 97)	4	15·5

* Ether-soluble acid – (ester glucuronide + glycine conjugate) from average values.

† R = the ratio glycine conjugate/ester glucuronide from average values. Values of R for benzoic acid and benzamide are 2·8 and 15·7 respectively (unpublished results).

‡ Dose 0·5 and 0·75 g.

Extraction of *o*-chloro- and *p*-fluoro-benzamide urines as collected (pH about 8·0) gave an amount of unchanged amide corresponding to less than 1 % of the dose fed: no amide was obtained by similar treatment of *p*-chlorobenzamide urine. There was no significant increase in the excretion of ethereal sulphate. About 2 years after these results had been obtained, the method for estimation of glycine conjugates was worked out. Two experiments were then performed with each compound in which the glycine conjugate as well as ether-soluble acid and glucuronide was estimated. This permitted the calculation of the ratio (glycine conjugate)/(ester glucuronide), R, and also of the amount of acid excreted unconjugated. These results are given in Table 2.

Nature of ether-soluble acid

The ether-soluble material extracted from metabolic urines, under the conditions used for the determination of ether-soluble acid, reduced alkaline cupric reagents and gave a positive naphthoresorcinol reaction, showing that ester glucuronides of the chloro- and fluoro-benzoic acids are soluble in ether. These could not, however, be isolated from the extracts or obtained in crystalline form via the usual lead procedure (cf. Bray *et al.* 1947).

Fractionation of the ether-soluble material by refluxing with successive small amounts of toluene (cf. Bray *et al.* 1947) led to the isolation, in every case, of the toluene-soluble halogen-substituted benzoic

Table 3. *Properties of chloro- and fluoro-hippuric acids isolated from metabolic urines*

Compound	Percentage of N		Melting point	
	Found	Calc.	Found	Literature
o-Chlorohippuric acid*	6·8	6·6	177–179°	176°†
m-Chlorohippuric acid	6·8	6·6	142°	143–144°†
p-Chlorohippuric acid	6·7	6·6	142–144°	143°†‡
o-Fluorohippuric acid	7·1	7·1	147°	121–121·5°§
m-Fluorohippuric acid	7·0	7·1	153°	152–153°§
p-Fluorohippuric acid	7·2	7·1	171°	161–161·5°§

* Found: Cl, 16·5; calc. 16·6%. † Novello *et al.* (1926). ‡ Hildebrandt (1903). § Coppola (1883).

acid, and except from *o*-fluorobenzoic acid urine, of the toluene-insoluble hippuric acid derivative. The properties of the hippuric acid derivatives are given in Table 3.

DISCUSSION

The results given in Tables 1 and 2 suggest that all the six amides studied are almost completely hydrolysed in the rabbit. This finding may be compared with the results previously obtained *in vitro*, which showed that the percentages of *o*-, *m*- and *p*-chloro- and *o*-, *m*- and *p*-fluoro-benzamides hydrolysed by rabbit-liver extracts in 5 hr. were 16, 42, 61, 59, 45, 46, respectively (Bray *et al.* 1950). From analogy with earlier comparisons (Bray *et al.* 1949*a*) it might have been expected that all but the first would be readily hydrolysed *in vivo*. The percentage hydrolysis *in vivo*, however, depends on the rate of excretion of the amide by the kidney, as well as on the rate of hydrolysis by the enzyme system. If administered amides are excreted only slowly, then they will be almost completely hydrolysed *in vivo* as long as the rate of hydrolysis is much greater than the rate of excretion unchanged. It appears that the chloro- and fluoro-benzamides are excreted unchanged very slowly, since the variations observed in hydrolysis *in vitro* are not revealed in the metabolic fate of those amides.

Similar considerations suggest that the proportion of the dose of an acid (or amide) excreted in conjugated form depends on the rate of excretion of the unconjugated acid and on the efficiency of the two conjugating enzyme systems. However, the ratio (R) of the amount of acid or amide excreted as the glycine conjugate to that excreted as the glucuronide will give the relative average rates of production of the two conjugates, whatever the rate of excretion of the free acid. It can be seen from Table 2 that in each case R is greater for an administered amide than for the corresponding acid. In a previous study of the metabolism of benzoic acid and its precursors (Bray *et al.* 1951) this variation in R was found to be a simple consequence of the kinetics of the conjugations. The rate of conjugation of benzoic acid with glucuronic acid is proportional to the body level of benzoic acid, whereas the rate of formation of hippuric acid is constant, so that R depends both on the dose level and on the rate of production of benzoic acid from a presursor. Since R is greater for the substituted amides than for the acids, it seems possible that the kinetics of the hydrolysis and conjugation reactions are similar to those of the unsubstituted acids and amides. We have previously reported that for benzoic, phenylacetic and *m*- and *p*-toluic acids and certain of their precursors (Bray *et al.* 1946; Bray, Thorpe & Wood, 1949*b*; Bray, Humphris & Thorpe, 1949) a greater proportion of glucuronide is excreted after administration of the free acid than after the precursor. Since after administration of these compounds the free acid is excreted to only a small extent and the value of R is greater for the amide than for the acid, the absolute amount of glucuronide produced is greater for the acid than for the amide. This will not necessarily be so when he unconjugated acid is readily excreted, as for the halogen-substituted benzoic acids, and in the absence of information on the kinetics of excretion of the unconjugated acids by the kidney it can only be concluded that R will be greater for amides than for acids, where the metabolic reactions follow kinetics of the same type as those found for benzoic acid and its precursors.

SUMMARY

1. The metabolism of the chloro- and fluorobenzoic acids and amides in the rabbit has been studied.

2. The acids are excreted unconjugated and conjugated with glucuronic acid and glycine to varying extents.

3. The amides are all almost completely hydrolysed, and the resulting acids are excreted partly free and partly conjugated. Traces of unchanged amide equivalent to less than 1% of the dose were isolated from *o*-chloro- and *p*-fluoro-benzamide urines.

4. The results obtained are discussed with reference to the kinetics of the reactions involved.

5. The substituted hippuric acids were isolated from the metabolic urines.

6. A method for the estimation of glycine in its conjugates in urine is described.

We wish to express our thanks to Miss B. G. Humphris for assistance with some of the analyses. The micro-analyses were carried out by Weiler and Strauss, Oxford and by Miss B. G. Humphris.

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The Effect of Heat on the Microbiological and Anti-anaemic Properties of Human Gastric Juice mixed with Vitamin B_{12}

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Ternberg & Eakin (1949) found in normal human gastric juice a substance which combined with vitamin B_{12} to make the vitamin unavailable as a growth factor in microbiological assays. The gastric juice of normal people was reported to contain more of the substance than the juice of patients with pernicious anaemia in relapse. It was suggested that this substance was the intrinsic factor of Castle. This suggestion aroused much interest, for, if confirmed, it could provide a relatively simple method for detecting intrinsic factor. Clinical observations by Hall *et al.* (1950) indicated that the complex formed between the intrinsic factor, extracted from hog stomach or duodenum, and vitamin B_{12}, was more stable to heat than the intrinsic factor alone. Ross (1950) found that substances having similar growth-promoting activity to that of vitamin B_{12} for *Euglena gracilis* were released on heating human serum. Heating at 100° for 30 min. released more activity than heating at 70° for the same time, so that serum apparently contains a complex of vitamin B_{12} which is fairly stable to heat.

During experiments to see whether certain proteins other than the substance in gastric juice could bind vitamin B_{12}, i.e. make it unavailable to bacteria, indications were obtained that the factor in gastric juice was more stable to heat in neutral than in acid solution. In view of present interest in the role of intrinsic factor in the metabolism of vitamin B_{12}, it seemed worth while to investigate the phenomenon further, using both microbiological and clinical methods.

EXPERIMENTAL AND RESULTS

The effect of acidity on the stability of the vitamin B_{12}-binding factor to heat

Treatment of gastric juice. The samples of gastric juice were residues from fractional test meals, obtained through the kindness of the Biochemistry Department of this hospital. Only samples containing free acid were used. The juice was filtered and stored in sterile flasks at 0° for at least 24 hr. After this treatment there was no evidence of bacterial growth when 0·5 ml. portions of the juice were added aseptically to broth and incubated for several days at 37°. The juice was neutralized just before use by adding sterile 2 N-NaOH drop by drop, and the pH was checked on a meter to between 7·2 and 7·6.

Microbiological assays. Vitamin B_{12} was assayed with *Lactobacillus leichmanii* in a medium containing acid-hydrolysed casein with the usual supplements. A solution of crystalline vitamin B_{12} (10 μg./ml., 'Cobione', Merck) was diluted for use as standard, and for addition to the gastric juice.

Sterile vitamin B_{12} solution was added to gastric juice in sterile graduated test tubes to give concentrations of 0·25 μg. or 0·5 μg./100 ml. of juice. After receiving the treatment indicated in Tables 1–3, the solutions were diluted with sterile water to give apparent vitamin B_{12} concentrations of 0·5 mμg./ml., and 1 ml. portions were added to the culture medium for assay. The amount of bacterial growth was read turbidimetrically after incubating at 37° for 18 hr.

In agreement with the results of Ternberg & Eakin (1949), gastric juice was shown to bind vitamin B_{12} (Table 1). The vitamin was released from its complex with the substance in gastric juice by heating the acid solution at 100° for 5 min. It was not necessary to incubate the juice and the vitamin together before adding them to the culture medium, in fact when vitamin B_{12} and gastric juice were added separately to the medium before incubation, very little bacterial growth occurred.

When gastric juice was neutralized before adding the vitamin B_{12}, heating at 100° for 5 min. no longer released all the vitamin from its bound form (Table 2). Neutralized gastric juice retained some capacity to bind vitamin B_{12} after heating at 100° for 15 min., but autoclaving at 20 lb. pressure for 30 min. destroyed the binding substance. This treatment also released the vitamin from its compound.

Microbiological activity was not released by heating a mixture of vitamin B_{12} and neutralized gastric juice, even when it was re-acidified with hydrochloric acid to the same pH as the original gastric juice after incubation. Autoclaving a re-acidified sample, however, released a considerable part of the vitamin B_{12}, although some was apparently destroyed. This would be expected, since experiments showed that over 95% of the pure vitamin was destroyed by autoclaving in acid solution. The stability to heat after re-acidification was not shown by mixtures which were stored at 0°

Table 1. *Effect of normal human gastric juice on the utilization of vitamin* B_{12} *by* Lactobacillus leichmanii

(Each reading of optical density is the mean of the values for four separate tubes containing identical samples. The optical densities of the cultures are related to the quantities of vitamin B_{12} added to the tubes by a curve, the slope of which decreases fairly rapidly in the range 0·0–1·0 mμg. vitamin B_{12}. Larger amounts of vitamin B_{12} only produce very small further increases in opacity.)

No.	Supplements present in 5 ml. culture medium	Optical density × 100 Exp. 1	Exp. 2	Exp. 4
1	Nothing	10	7	11
2	0·5 mμg. vitamin B_{12}	58	60	43
3	0·5 mμg. vitamin B_{12} + 0·1 ml. gastric juice, incubated 22 hr. at 37° before adding to medium	14	9	12
4	As no. 3 but with 0·2 ml. juice	—	12	10
5	As nos. 3 and 4, but heated 5 min. at 100° after incubation and before adding to medium	64	66	57
6		—	64	66
7	0·5 mμg. vitamin B_{12} + 0·05 ml. gastric juice added separately to medium just before inoculation	10	—	—

Table 2. *Effect of acidity on the breakdown by heat of the compound formed by vitamin* B_{12} *with a substance in gastric juice*

(All tubes in nos. 3–12 were incubated 22 hr. at 37° and heated 5 min. at 100° before adding to culture medium. Nos. 13 and 14 were autoclaved 30 min. at 20 lb. after incubating 22 hr. at 37°.)

No.	Supplements present in 5 ml. culture medium	Optical density × 100 Exp. 3	Exp. 4	Exp. 5	Exp. 6
1	Nothing	7	11	9	7
2	0·5 mμg. vitamin B_{12}	42	43	47	43
3	0·5 mμg. vitamin B_{12} + 0·1 ml. acid gastric juice	56	57	66	52
4	0·5 mμg. vitamin B_{12} + 0·2 ml. acid gastric juice	64	66	70	56
5	0·5 mμg. vitamin B_{12} + 0·1 ml. neutralized gastric juice	10	10	18	10
6	0·5 mμg. vitamin B_{12} + 0·2 ml. neutralized gastric juice	8	7	14	8
7	As nos. 5 and 6, but re-acidified after incubation	—	10	—	10
8		—	6	—	10
9	As nos. 5 and 6, but neutralized juice heated 15 min. at 100° before incubation	10	10	36	8
10		10	—	30	8
11	As nos. 5 and 6, but neutralized juice autoclaved 30 min. at 20 lb. before incubation	54	—	62	48
12		56	—	68	56
13	0·5 mμg. vitamin B_{12} + 0·1 ml. neutralized gastric juice	—	50	61	44
14	0·5 mμg. vitamin B_{12} + 0·2 ml. neutralized gastric juice	—	61	70	48
15	As nos. 13 and 14, but re-acidified before autoclaving	—	39	—	26
16		—	42	—	28

Table 3. *Effect of incubation on the stability to heat in acid solution of the compound of vitamin B_{12} with a substance in gastric juice*

(*A*, vitamin B_{12} and gastric juice mixed and stored 22 hr. at 0° and then heated 5 min. at 100°; *B*, vitamin B_{12} and gastric juice mixed and incubated 22 hr. at 37° and then heated 5 min. at 100°.)

No.	Supplements present in 5 ml. culture medium	Optical density × 100			
		Exp. 7		Exp. 8	
1	Nothing	27		4	
2	0·5 mμg. vitamin B_{12}	57		43	
		A	*B*	*A*	*B*
3	0·5 mμg. vitamin B_{12} + 0·1 ml. acid gastric juice	66	66	54	53
4	0·5 mμg. vitamin B_{12} + 0·2 ml. acid gastric juice	70	68	58	61
5	0·5 mμg. vitamin B_{12} + 0·1 ml. neutralized gastric juice	19	22	4	4
6	0·5 mμg. vitamin B_{12} + 0·2 ml. neutralized gastric juice	18	20	4	3
7	As nos. 5 and 6, but re-acidified with HCl after incubation or storage at 0°	66	51	47	8
8		70	42	42	5

instead of being incubated, although incubation was not essential for the greater stability in neutral solution (Table 3). Perhaps, therefore, incubation produces some change in the vitamin B_{12}-binding factor which makes it more stable to heat in acid solution.

The somewhat higher growth responses shown in the tables for mixtures of gastric juice and vitamin B_{12}, as compared with those due to the vitamin alone, are due to the small amounts of growth-promoting substances present in the gastric juice itself.

Thus the substance in gastric juice which combines with vitamin B_{12}, and the compound so formed, appear to be more stable to heat in neutral solution than in solutions at the pH of normal human gastric juice. If neutralized gastric juice and vitamin B_{12} are first incubated together, the compound appears to retain its stability to heat, even on re-acidification to the original pH of the gastric juice.

Failure of a microbiologically inactive compound of vitamin B_{12} to show haemopoietic acitivity in pernicious anaemia

It has been suggested (Ungley, 1950) that Castle's intrinsic factor may act by combining with vitamin B_{12} to make it unavailable for the growth of intestinal bacteria; otherwise the bacteria would use up the vitamin so that it could not be absorbed by the host. Failure to secrete the intrinsic factor, such as occurs in patients with pernicious anaemia, is thus supposed to cause a deficiency of vitamin B_{12} in the host. On this hypothesis, a mixture of neutralized gastric juice and vitamin B_{12}, which has been incubated at 37° and then heated at 100°, should retain anti-anaemic activity when given by mouth to a patient with pernicious anaemia in relapse, since most of the vitamin B_{12} would be in a form unavailable to micro-organisms.

Accordingly, a number of samples of gastric juice from normal subjects, either resting samples or those secreted under histamine stimulation, and all containing free acid, were pooled and stored at 0° and pH 2·0. A patient with classical Addisonian pernicious anaemia in relapse was given 5 μg. of vitamin B_{12} ('Cytamen', Glaxo) in 100 ml. water, daily by mouth for 10 days. For the following 11 days he received 100 ml. of neutralized gastric juice, which had been incubated 22 hr. at 37° with 5 μg. vitamin B_{12} and then heated at 95° for 20 min. In a third period of 10 days he was given a similar mixture of gastric juice and vitamin B_{12} which had been incubated but not heated at 95°. All the samples of gastric juice were portions of the pooled specimen, and they were neutralized with 10N-NaOH just before starting incubation.

Table 4 shows that a considerable proportion of the vitamin B_{12} remained in a bound form after heating at 95° for 20 min. The patient did not respond either to 5 μg. vitamin B_{12} per day alone, or to a mixture of the vitamin and gastric juice which had been incubated and heated. There was a significant response to the vitamin incubated with unheated gastric juice (Table 5). Similar results were obtained by Hall (1950), who found that intrinsic factor in gastric juice was destroyed by incubating neutralized juice at 25° for 24 hr., followed by heating at 70° for 1 hr.; incubating at 25° without heating did not destroy the factor.

DISCUSSION

The results show that when neutralized human gastric juice is mixed with vitamin B_{12} and heated at 100°, a considerable part of the vitamin remains in a form which cannot be used as a growth factor by bacteria, whereas the power of the juice to potentiate the haemopoietic action of vitamin B_{12} by mouth is lost. Thus Castle's intrinsic factor seems to be destroyed by a degree of heating which is resisted by the substance which reacts with vitamin B_{12}, indicating that the two factors in gastric juice

Table 4. *Effect of heat on mixtures of neutralized gastric juice and vitamin* B_{12}

Additional supplements	Vitamin B_{12} in 5 ml. culture medium (mμg.) 1·0	2·0	3·0	4·0
	Optical densities (× 100) with the vitamin B_{12} and the additional supplements			
Nothing	64	64	66	65
0·1 ml. neutralized gastric juice, incubated 22 hr. at 37° with the vitamin B_{12} before adding to medium	30	47	54	62
0·1 ml. neutralized gastric juice, incubated 22 hr. at 37° with the vitamin B_{12} and then heated 20 min. at 95° before adding to medium	19	26	40	48
0·1 ml. 0·067 M-phosphate buffer pH 7·4, incubated 22 hr. at 37° with the vitamin B_{12} and then heated 20 min. at 95° before adding to medium	71	73	74	76

Table 5. *Response of an untreated patient with pernicious anaemia to mixtures of vitamin* B_{12} *and gastric juice by mouth*

Period no.	Daily treatment	No. of days observed	Red blood cell count (millions/cu.mm.) Beginning	End	Haemoglobin (g./100 ml.) Beginning	End	Maximum reticulocytosis (%)	Day observed
1	5 μg. Vitamin B_{12} in 100 ml. water	10	1·29	1·08	6·4	5·4	2·2	5
2	5 μg. Vitamin B_{12} in 100 ml. neutralized gastric juice, incubated 22 hr. at 37° and then heated 20 min. at 95°	11	1·08	1·07	5·4	4·6	3·2	4
3	5 μg. Vitamin B_{12} in 100 ml. neutralized gastric juice, incubated 22 hr. at 37°	14	1·07	2·03	4·6	8·0	10	11

are not identical. These experiments do not preclude the possibility that the substance which binds vitamin B_{12} may be one of several factors involved in the absorption of vitamin B_{12} from the gastrointestinal tract, and which together constitute the intrinsic factor.

SUMMARY

1. The factor in normal human gastric juice which combines with vitamin B_{12} so that the latter is not available as a growth factor for microorganisms, is shown to be more stable to heat in neutral solution than in acid solution.

2. Heating mixtures of gastric juice and vitamin B_{12} under conditions which left a considerable part of the vitamin in a form unavailable to bacteria, destroyed the ability of the juice to potentiate the action of orally administered vitamin B_{12} in a patient with untreated pernicious anaemia.

3. These results suggest that the vitamin B_{12}-binding factor is not identical with the intrinsic factor of Castle.

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The Effects of Glucose and Potassium on the Metabolism of Pyruvate in *Lactobacillus arabinosus*

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During an investigation of the decarboxylation of L-malate by cells of *Lactobacillus arabinosus*, 17–5 (Nossal, 1951*a*) it was observed that pyruvate also yielded carbon dioxide. Like the decarboxylation of malate, the decomposition of pyruvate was found to be most rapid in the presence of glucose, potassium chloride and manganous chloride. Under most conditions the decomposition of pyruvate in *Lb. arabinosus* was found to be in agreement with the equation:

$$\underset{\text{pyruvic acid}}{2CH_3.CO.COOH} = 2CO_2 + \underset{\text{acetoin}}{CH_3.CO.CHOH.CH_3}$$

This reaction is known to occur in numerous bacteria, e.g. *Clostridium acetobutylicum* (Wilson, Peterson & Fred, 1927), *Aerobacter aerogenes* (Silverman & Werkman, 1941), *Staphylococcus aureus* (Watt & Krampitz, 1947), *Bacillus subtilis* (Lemoigne, Hooreman & Croson, 1949), *Micrococcus pyogenes*, var. *aureus* (Watt, 1949), and *Lactobacillus plantarum* (Rowatt, 1949*a*, *b*). Aerobically, in the absence of glucose, the amount of pyruvate used was not wholly accounted for by the amount of acetoin formed. Part of this work has been communicated to the Biochemical Society (Nossal, 1951*b*).

METHODS

Suspensions of Lactobacillus arabinosus, 17–5. Directions for maintaining subcultures, growing bulk supplies and preparing washed suspensions of *Lb. arabinosus*, 17–5, have been described previously (Nossal, 1951*a*). Addition of 0·1 M-pyruvate to the growth medium did not increase the rate of pyruvate decomposition by the bacteria; malate-adapted cells (Korkes & Ochoa, 1948) were slightly more active than normal cells, and retained their activity better on storage; therefore 0·1 M-DL-malate was usually included in the growth medium. Centrifuged cells were suspended in 5 vol. of 0·1 M-KCl for storage and kept at 2°.

Analytical procedures. Pyruvate was estimated by the carboxylase method of Krebs & Johnson (1937). Preparations of carboxylase lost much of their activity after 1 week's storage at 2°, but if kept at −12° they were still fully active after 10 weeks. Acetoin was estimated by the Voges-Proskauer reaction (see Eggleton, Elsden & Gough, 1943; Westerfeld, 1945). Since none of the constituents of the experimental mixtures interfered with the estimation or gave rise to acetoin during the estimation, portions of samples to be estimated were pipetted directly into a mixture of α-naphthol in alkali and saturated aqueous creatine (see Eggleton *et al.* 1943) without preliminary deproteinization, distillation or oxidation. After filtration and allowing 60–90 min. for colour development, the optical densities were measured in a Beckman spectrophotometer at 535 mμ. On this instrument, the optical density was proportional to the concentration of acetoin only up to a level of 50 μg. acetoin (not 100 μg. as in the procedure of Eggleton *et al.* 1943), and samples were accordingly diluted to fall within this range. Standard solutions of acetoin were prepared from the distillate of a commercial sample of acetyl methyl carbinol (Light and Co.) boiling at 140–143° (corr.). These were compared spectrophotometrically with a solution of diacetyl, standardized gravimetrically as the nickel dimethyl glyoxime (Niel, 1927), and were found to be 96–100% pure. Three standards of acetoin (equivalent to 12·5, 25 and 50 μg.) were included in each set of estimations.

Measurement of the rate of pyruvate decomposition. The rate of pyruvate decomposition under varying conditions was followed at acid pH by manometric measurement of the rate of CO_2 evolution in standard Warburg equipment. The bath temperature used throughout was 30°. The gas phase contained air or N_2; in anaerobic experiments, sticks of yellow phosphorus were placed into the centre wells of the vessels to ensure complete removal of O_2; in aerobic experiments, O_2 uptakes were measured in vessels containing 0·2 ml. 2N-NaOH and fluted filter paper in the centre well. Q values are given as μl. O_2 (or CO_2)/mg. dry wt. cells/hr.

RESULTS

Identification of acetoin as a product of pyruvate decomposition

After bacterial suspensions had acted on pyruvate under optimum conditions (see below), the distillate from the reaction mixture gave the Voges-Proskauer test for diacetyl and acetoin. Some tests distinguishing between acetoin and diacetyl are given in Table 1, from which it can be seen that the product of pyruvate decomposition behaved like acetoin; after oxidation with ferric chloride-ferrous sulphate mixture (Westerfeld, 1945), which is known to convert acetoin to diacetyl, the product of pyruvate decomposition gave the reactions of diacetyl. The osazones prepared from acetoin, diacetyl and the reaction product were identical (melting points and mixed melting points all falling within the range 237–242°, corrected).

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Table 1. *Differentiation between acetoin, diacetyl and the product of pyruvate decarboxylation*

(The contents of several Warburg vessels were pooled after completion of an experiment (complete system as in Table 2) and distilled almost to dryness to remove the volatile product from residual glucose and pyruvate. This distillate is the 'product' referred to below. A portion of the distillate was oxidized according to Westerfeld (1945) and the mixture distilled again. This second distillate is referred to as the 'oxidized product'.)

	Voges-Proskauer reaction		Formation of			
	Spectrum (absorption peak) (mμ.)	Time for development of maximum colour (min.)	Ni dimethyl-glyoxime	Semicarbazide	Phenylhydrazone	2:4-Dinitrophenyl-hydrazone
Product Acetoin	520	60	–	Only after concentrating reaction mixture	Begins to form at 70°	Begins to form at 70°
Oxidized product Diacetyl	530	7–10	+	Forms readily at room temperature	Forms immediately at room temperature	Forms immediately at room temperature

Effect of glucose, potassium chloride and manganous chloride, on the anaerobic decomposition of pyruvate. Maximal rates of CO_2 evolution with pyruvate as the substrate were observed anaerobically in the presence of added glucose, KCl and $MnCl_2$ (Table 2); the effect of KCl is less pronounced

Table 2. *Effect of glucose,* KCl *and* $MnCl_2$ *on the anaerobic decomposition of pyruvate*

(Complete system contains 6 mg. dry wt. freshly harvested cells, 0·025 M-$MnCl_2$, 0·1 M-KCl, 0·01 M-glucose, 0·05 M-citrate-phosphate buffer pH 4·0, 0·01 M pyruvate. Gas: N_2. Data in μmol. Experimental period: 60 min.)

Notes	Pyruvate utilized	CO_2 evolved	Acetoin formed
Complete system	41	42	21
No glucose	16	14	8
No KCl	37	35	18
No $MnCl_2$	25	24	11

than in the decarboxylation of L-malate (Nossal, 1951*a*); unless the cells are freshly harvested, the anaerobic decomposition of pyruvate is very slow in the absence of glucose (see Table 7). The amount of pyruvate decomposed is almost totally accounted for by the amounts of acetoin and CO_2 formed.

Variations in the behaviour of cell suspensions towards pyruvate in the presence of oxygen. Although grown under apparently identical conditions, some washed suspensions of *Lb. arabinosus* showed a considerable oxygen uptake with pyruvate as substrate (Q_{O_2} about 50), whereas others had a lower Q_{O_2} (about 10 to 20), but the Q_{CO_2} values in air or nitrogen did not differ appreciably; the variations occurred, not at random, but in cycles, both variants being approximately equally distributed. The magnitude of the effect of glucose on the rate of pyruvate decomposition in the presence of oxygen varied inversely as the Q_{O_2}. In suspensions having a high Q_{O_2} value neither glucose nor KCl increased the rate of aerobic CO_2 evolution from pyruvate (Table 3), but in cells having a low Q_{O_2}, the same effects of glucose and KCl were observed as those found when pyruvate decomposition was tested anaerobically.

It seemed possible that variations in growth conditions and the composition of the medium were responsible for the variability of the oxygen uptake. However, it will be seen from Table 4 that partial deficiencies of pantothenic acid, nicotinic acid and biotin did not cause variations in either Q_{O_2} or Q_{CO_2}; the growth period, age of the medium, and inclusion

Table 3. *Aerobic conversion of pyruvate to acetoin*

(The complete system contains 4 mg. dry wt. cells, 0·0025 M-glucose, 0·025 M-$MnCl_2$, 0·05 M-KCl, 0·05 M-citrate-phosphate buffers, 0·01 M-pyruvate. Experimental period: 60 min. Gas: air. Data in μmol. $Q_{O_2} = \mu l.\ O_2$/mg. dry wt. cells/hr.)

Age of cell suspension (days)	Q_{O_2}	pH	Notes	Pyruvate utilized	CO_2 evolved	Acetoin formed
5·5	48	4·0	Complete system	41	42	20
			No glucose	41	41	15
			No KCl	42	41	18
			No $MnCl_2$	37	38	17
1	15	5·0	Complete system	28	29	13
			No glucose	15	17	4
			No KCl	19	19	6
			No $MnCl_2$	22	22	10

Table 4. *Effects of variations in growth conditions on the aerobic utilization of pyruvate by* Lb. arabinosus

(Normal growth conditions given under Methods; all cell suspensions tested in the complete system as described in Table 3. Q_{O_2} (or Q_{CO_2}) = μl. O_2 (or μl. CO_2)/mg. dry wt. cells/hr.)

Exp.	Yield of cells (mg. dry wt./100 ml. medium)	Growth temperature (°)	Medium	Q_{O_2}	Q_{CO_2}
1	183	30	Normal	46	210
	155	38	Normal	19	90
	177	38	Glucose added to medium before sterilization	34	185
2	198	30	Added 10^{-6} M-pantothenic acid	39	214
	206	30	Sterilized at pH 4·5 (to partly remove pantothenic acid)	47	208
	205	30	As above, added 10^{-6} M-pantothenic acid	48	189
3	250	30	Normal; fresh	37	224
	280	30	Normal; 6 weeks old	49	218
4	—	38	Nicotinic acid deficient*	60	252
5	—	38	Biotin deficient†	40	205
6	—	30	Normal; growth period: 12 hr.	11	116
			growth period: 16 hr.	24	172
			growth period: 20 hr.	30	200
			growth period: 24 hr.	37	200
			growth period: 36 hr.	21	147

* Hughes & Williamson (1949).

† Nicotinic acid assay medium (Barton-Wright, 1946) with an old solution of biotin added; poor growth. Normal growth could be restored by addition of 0·4 μg. biotin.

of glucose in the medium during sterilization (instead of sterilizing the glucose separately and adding it at the start of the experiment) also caused no marked differences in the ratio Q_{O_2}/Q_{CO_2}; when grown at 38° instead of 30°, both Q_{O_2} and Q_{CO_2} values were reduced to approximately the same extent. The reason for the observed variations in the aerobic behaviour of different cell suspensions therefore remains obscure. Bacteriological tests on different cell suspensions showed that the variations were not due to contamination by other bacteria.

Table 5. *Effect of glucose concentration on the rate of* CO_2 *evolution from pyruvate*

(6 mg. dry wt. cells (age of suspension 2 days); 0·025 M-$MnCl_2$, 0·1 M-KCl, 0·05 M-citrate-phosphate buffer pH 4·0; 0·01 M-pyruvate. Gas: air.)

Glucose (final concn. M)	Maximum rate of CO_2 evolution (μl./5 min.)	Total CO_2 evolved in first hr. (μl.)
—	35	336
4×10^{-5}	60	500
$1·6 \times 10^{-4}$	116	619
$6·2 \times 10^{-4}$	159	706
$2·5 \times 10^{-3}$	182	791
10^{-2}	167	790

Catalytic effect of glucose. Both aerobically and anaerobically, the effect of glucose on the rate of CO_2 evolution from pyruvate is catalytic. In the experiment shown in Table 5, addition of 0·16 μmol. glucose (the lowest concentration tested) increased CO_2 evolution by 8 μmol. in the first hour. No CO_2 evolution occurred when glucose was the only substrate.

Glucose depressed the oxygen uptake with pyruvate as the substrate. Aerobically, the yield of acetoin from pyruvate as the only substrate was not quantitative, but as low as 50% of the expected value (see Table 3, Exp. 2). The yield became quantitative in the presence of glucose. The second aerobic pathway of pyruvate decomposition has not been examined.

Effects of various substances on pyruvate decomposition and acetoin formation. Fructose accelerated the rate of CO_2 evolution from pyruvate, and increased the amount of acetoin formed, about half as effectively as equivalent amounts of glucose. Galactose and DL-glyceraldehyde had a slight effect. At pH 5·0, $MnCl_2$ accelerated the rate of CO_2 evolution twice as effectively as $MgCl_2$, but at pH 4·0 both were equally effective. RbCl was as effective as KCl, and CsCl was slightly less effective. Similar observations have been reported earlier for the decarboxylation of L-malate (Nossal, 1951*a*).

Glucose, fructose, galactose, glyceraldehyde, lactate and acetaldehyde did not form acetoin in the absence of pyruvate; lactate and acetaldehyde did not increase acetoin formation from pyruvate.

Inorganic phosphate (0·05 M) and ATP (0·0025 M) did not affect either the rate of CO_2 evolution or acetoin formation.

Effect of potassium ions on the rate of fermentation of glucose

Because of the connexion between the effects of glucose and KCl in the decomposition of pyruvate, the effect of KCl on the rate of acid production from glucose was tested. Acid production was measured manometrically in 0·013 M-sodium bicarbonate and 100 % CO_2, giving a pH of 6·0. It was found that addition of 0·1 M-KCl increased the rate of acid production from glucose by 60–100 % and reduced the lag period from 20 to 25 min. to 5 min. Thus potassium ions have a similar effect on acid production from glucose in *Lb. arabinosus* as on glucose fermentation by yeasts (Farmer & Jones, 1942).

Effect of buffer composition and of pH. Pyruvate decomposition was more rapid in 0·05 M-citrate-phosphate buffers than in 0·05 M-acetate buffers of the same pH: at pH 5·0, the Q_{CO_2} values were 164 in citrate-phosphate buffer and 66 in acetate buffer; at pH 4·0, Q_{CO_2} values were 390 in citrate-phosphate buffer and 300 in acetate buffer; virtually no decomposition occurred in 0·05 M-phthalate buffers.

Utilization of pyruvate proceeded at a high rate only within the pH limits 3·4–5·5. Aerobically the pH optimum was 4·6, anaerobically it was 3·7 (Table 6).

Table 6. *Effect of* pH *on the rate of decarboxylation of pyruvate*

(Complete system in all vessels as in Tables 2 and 3.)

Buffer pH	Gas	Lag period (min.)	Max. rate of CO_2 evolution (μl./5 min.)
	N_2		
3·2		30	8
3·5		20	112
3·7		10	148
4·0		10	139
4·2		10	110
4·5		10	89
4·9		15	49
	Air		
3·4		25	36
3·8		15	66
4·0		10	120
4·5		10	180
4·7		10	180
5·1		15	112
5·5		20	50

The pH is liable to change in the course of the experiments. At the high rates of decarboxylation observed around pH 4, the pH rises on account of the base released by decarboxylation, and at pH 5 glucose is so rapidly converted to lactic acid that the pH drops. The data of Table 6 are taken from initial rates of decarboxylation, during which the pH of the experimental mixture was near that of the buffers added. At all pH levels the amount of pyruvate used was quantitatively accounted for by acetoin formation (as in the complete systems of Tables 2 and 3) in the presence of added glucose, but, aerobically, not in its absence.

Loss of activity on storage. Rowatt (1950) observed that cells of *Lb. plantarum* rapidly lost the ability to metabolize pyruvate anaerobically on storage at 4°. This is also true for *Lb. arabinosus*, but only when the activity was tested anaerobically without added glucose (Table 7); therefore the cells

Table 7. *Loss of activity on storage*

(Complete system as in Tables 2 and 3, omitting glucose, 4 mg. dry wt. cells. Experimental period 60 min. Cells in Exp. 1 malate-adapted, in Exp. 2 grown in absence of malate.)

Exp.	Duration of storage (days)	Glucose added (0·005 M)	Cells stored in	Gas phase	Total CO_2 evolved (μl.)
1	0	+	—	N_2	790
	0	−	—	N_2	356
	0	+	—	Air	842
	0	−	—	Air	740
	5	+	H_2O	N_2	654
	5	−	H_2O	N_2	50
	5	+	0·1 M-KCl	N_2	794
	5	−	0·1 M-KCl	N_2	37
	5	+	0·1 M-KCl	Air	884
	5	−	0·1 M-KCl	Air	825
2	0	+	—	N_2	753
	0	−	—	N_2	327
	0	+	—	Air	788
	0	−	—	Air	715
	2	+	H_2O	N_2	740
	2	−	H_2O	N_2	70
	2	+	H_2O	Air	800
	2	−	H_2O	Air	720
	4	+	H_2O	N_2	462
	4	−	H_2O	N_2	20

did not lose the ability to metabolize pyruvate, but rather to metabolize it in the absence of glucose or O_2. The ability to use pyruvate in the presence of glucose or O_2 was retained longer in 0·1 M-KCl than in water.

DISCUSSION

Effects of glucose on acetoin formation from pyruvate have been noted by other workers: Watt (1949) showed that *Micrococcus pyogenes* var. *aureus* formed acetoin from pyruvate only when grown on glucose-containing media. Rowatt (1949*a*, *b*) described an initial increase in Q_{CO_2} and a reactivation of inactivated cells when glucose was added to *Lb. plantarum* acting on pyruvate. However, the inhibition by glucose of oxygen uptake with pyruvate as the substrate does not seem to have been found previously. The effect of glucose in *Lb. arabinosus* is twofold: it markedly accelerates anaerobic decomposition of pyruvate (the products of which are

solely acetoin and carbon dioxide), and aerobically it increases acetoin formation. Catalytic effects of glucose have been described for other oxido-reduction systems, e.g. the formic hydrogenlyase of *Bacillus coli* (Lascelles, 1948) and the decarboxylation of L-malate in adapted cells of *Lb. arabinosus* (Nossal, 1951*a*), but the mechanism of these effects remains obscure.

An influence of potassium on acetoin formation has not been described hitherto. In the system studied here, the effects of glucose and potassium are interlinked: potassium accelerates pyruvate decomposition and increases acetoin formation only in experiments where glucose also has an effect (compare Exps. 1 and 2, Table 3). Since potassium increases glucose fermentation in *Lb. arabinosus*, its action could be accounted for by acceleration of glucose metabolism. In this respect the effect differs from that observed in the decarboxylation of malate by the same organism: the increases in activity caused by potassium in the malate system are independent of glucose; furthermore, the potassium effect is also found in cell-free, partially purified extracts of 'malic enzyme' from *Lb. arabinosus* (Korkes, del Campillo & Ochoa, 1950, footnote, p. 898); since these extracts are unlikely to contain or to be able to metabolize glucose, the effect of potassium in the decarboxylation of malate can hardly be related to glucose metabolism.

Potassium has also been found to accelerate the oxidative decarboxylation of malate (Lwoff & Ionesco, 1947) and the decarboxylation of oxaloacetate (Lwoff & Ionesco, 1948) in *Moraxella Lwoffi*, but no effects of glucose are described.

There is no evidence that *Lb. arabinosus* grown on the media used for this work converts pyruvate anaerobically to lactate, acetate and carbon dioxide, as suggested by Blanchard, Korkes, del Campillo & Ochoa (1950). It is possible that growth on the different media employed by Blanchard *et al.* modified the pathway of pyruvate decomposition.

SUMMARY

1. Anaerobically, washed suspensions of *Lb. arabinosus* require glucose, potassium chloride and manganous chloride for maximum rates of pyruvate decomposition. The amount of pyruvate utilized is accounted for by formation of carbon dioxide and acetoin.

2. Aerobically, different batches of cell suspensions differ in their Q_{O_2} values with pyruvate as substrate, the Q_{O_2} varying from 10 to 50. Cells with high Q_{O_2} values require only addition of manganous chloride for the maximum rates of pyruvate utilization; cells with low Q_{O_2} values require glucose, potassium chloride and manganous chloride for full activity. Glucose acts catalytically, depresses oxygen consumption and increases acetoin formation. Potassium chloride is effective only in cells which also require glucose for maximum activity.

3. Fructose and galactose accelerate pyruvate utilization and increase acetoin formation, but less effectively than glucose. No acetoin is formed from sugars, glyceraldehyde, lactate or acetaldehyde: lactate and acetaldehyde do not increase acetoin formation from pyruvate.

4. Pyruvate utilization is more rapid in citrate-phosphate buffers than in acetate buffers of the same pH; phthalate inhibits strongly. Aerobically, the pH optimum of the reaction is about 4·6, anaerobically about 3·7.

5. Cell suspensions rapidly lose their ability to decompose pyruvate anaerobically without added glucose; the ability to decompose pyruvate aerobically, or anaerobically in the presence of glucose, is retained for several days at 2°, especially when the cells are suspended in 0·1 M-potassium chloride.

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The *Striga* Germination Factor

2. CHROMATOGRAPHIC PURIFICATION OF CRUDE CONCENTRATES*

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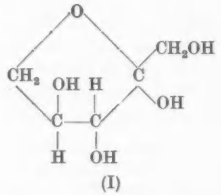
In the first paper of this series (Brown, Johnson, Robinson & Todd, 1949*a*), the purpose of which is the determination of the chemical nature of the stimulant liberated from actively growing roots and responsible for the germination of seeds of the angiospermous parasite, *Striga hermonthica*, the preparation of crude concentrates was described as well as a method for biological assay. It was found that the stimulant could be adsorbed on to activated charcoal and eluted with 70 % aqueous acetone and that the material thus obtained contained carbohydrates including free pentoses. On testing a wide variety of sugars for their effects in the germination of *Striga* seeds, a powerful stimulation was obtained with dilute solutions of D-xyloketose (I), the maximum germination being recorded at a concentration of 1×10^{-6} mg./ml., and some stimulation at 1×10^{-7} mg./ml.



(I)

D-Xyloketose resembled the natural stimulant in two other important respects. In the first place the biological activities of dilute aqueous solutions of both disappeared within 72 hr. on keeping at 15° or within 1 hr. at 100° and, secondly, it was found (Brown, Johnson & Robinson, 1949*b*) that the substance released from the roots of the host, e.g. *Sorghum vulgare*, and D-xyloketose both stimulated root extension. It therefore seemed very probable that the stimulating action of the concentrates obtained from the exudates of the host roots was either similar to, or identical with, D-xyloketose. Certain other sugars, e.g. D-fructose, D-fructose 1:6-diphosphate and L-sorbose, promoted slight germination with *Striga* seeds, but no substances other than carbohydrates gave any such stimulation within the same concentration range and time as was given by the natural stimulant.

The work on the germination of *Striga* seed has been continued along two lines. The action of sugars has been examined further and this will be reported in a separate paper. The present communication deals with some of the work concerned with the isolation and concentration of the stimulant itself.

Paper-strip chromatography has been applied to the study of the compositions of the crude concentrates and also to the determination of the distribution of active substances on the chromatograms. An examination of the carbohydrates present revealed a pentose with chromatographic properties very similar to arabinose, but no xyloketose has ever been detected in any of the samples tested. Besides arabinose, four other carbohydrates were generally present. One of these was identified as D-glucose, previously isolated as the *o*-nitrophenylhydrazone (Brown *et al.* 1949*a*) and another possibly as 2-ketogluconic acid and the others which were not identified were probably di- or poly-saccharides. At the same time, five or six amino compounds were also shown to be present in traces by the ninhydrin reaction, and by direct comparison with standards, three of these were shown to have properties closely related to norleucine (or leucine), valine and tyrosine. A full investigation of the nature of these carbohydrates and amino-acids was deferred when other chromatograms were examined for the location of biologically active material by the method previously described (Brown, Greenwood, Johnson, Long & Tyler, 1951*b*). A chromatogram obtained with the crude fluid in which roots of *Sorghum* had been growing showed four separate zones of activity, two of these being in a region in which carbohydrates occur and one immediately behind the solvent front (Fig. 1). Moreover, it was evident that this last component, which was not a simple carbohydrate or amino-acid, was probably the most important constituent of the complex. Chromatograms of acetone-ether eluates of the crude stimulant showed much the same distribution, although the contribution of the material which travelled close to the solvent front was relatively greater in these cases (Fig. 2). The difference between the two chromatograms

* An earlier paper by Brown *et al.* (1949*a*) is taken to be Part 1 of this series.

suggested that the elution had involved a greater concentration of the component with the high R_F value.

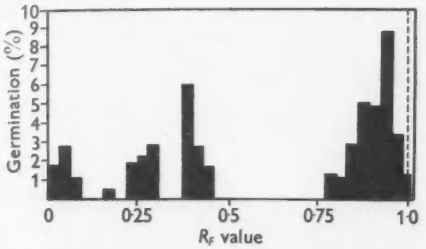


Fig. 1. Paper-strip chromatographic analysis of crude *Striga* germination factor.

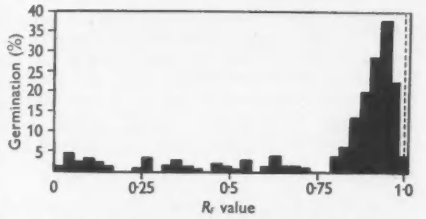


Fig. 2. Paper-strip chromatographic analysis of crude *Striga* germination factor after extraction with acetone-ether.

These observations formed the basis of the subsequent development of the investigation which has been directed towards the isolation of the active material which on the chromatograms was located immediately behind the solvent front. Although little is known at the present time of the detailed chemical structure of this substance, the investigation has revealed that in many respects it is very similar to the stimulant involved in the germination of *Orobanche* seeds (Brown, Greenwood, Johnson & Long, 1951*a*), although, whereas the *Orobanche* material is relatively stable in acid solutions (Brown *et al.* 1951*b*), solutions of the *Striga* stimulant are not. Both germination factors are very unstable in alkaline solution. The techniques employed in the purification of the *Orobanche* stimulant have also been used in the present work.

In the earlier work on the isolation of the *Striga* germination factor (Brown *et al.* 1949*a*), 70% aqueous acetone was used for the elution of the stimulant from the charcoal, but this solvent has now been replaced by 75% acetone-ether as a result of a comparative study of a range of eluting solvents (Table 1). All solvent evaporations were carried out under reduced pressure at temperatures $< 35°$ in an atmosphere of nitrogen, and the pH of the solutions was maintained at 5·5–6·5 by the addition of small quantities of sodium bicarbonate solution, where necessary. By this means, crude concentrates were obtained which varied in appearance between pale-yellow amorphous solids and colourless glasses depending on the inorganic content, and which had activity values (Brown *et al.* 1949*a*) ranging from 1000 to 50,000 on the arbitrary scale.

The second stage in the purification has been an extraction with an organic solvent, and of those investigated (Table 3), diethyl ether is the most satisfactory and this step has been included in all the later preparations. It was frequently observed during these extractions that the percentage recovery of activity exceeded 100 and a similar effect was also encountered in the *Orobanche* series. It is attributed partly to the retention of substances in the aqueous phase which inhibit the germination of the *Striga* seeds, and partly to inaccuracies in the method of biological assay.

For the further purification of the germination factor various methods have been investigated, including fractional distillation at very low pressures, chromatography and counter-current extraction, the last largely forming the subject of a later paper. Although the distillation method caused considerable deactivation and decomposition, it was possible, nevertheless, to obtain an intermediate fraction with a considerably enhanced activity (1×10^6), and this result is of significance in view of the relative instability of the compound in aqueous solution. The active fraction from the distillation was subjected to a bath temperature of 60–115° for 4–5·5 hr., whereas an aqueous solution of the stimulant loses its biological activity completely after 1 hr. when heated at 100°.

In view of the success of the paper-strip chromatograms in separating the active material from sugars and amino-acids, chromatography on cellulose appeared to be an attractive possibility for the purification of the concentrates on a scale large enough to provide material for chemical studies. Alumina was not used as this had proved unsatisfactory in the case of the *Orobanche* stimulant. Water-saturated *n*-butanol was the preferred solvent for use with the cellulose columns as less biological deactivation was experienced in the absence of acetic acid. The stimulant was thus eluted in a sharply defined zone and, after removal of the solvent, material was obtained with activity values in the order 1×10^6 to 1×10^7.

EXPERIMENTAL

Adsorption of biologically active material on charcoal

The technique adopted was essentially the same as that described previously (Brown *et al.* 1949*a*). Seedlings of the host plant, *Sorghum vulgare*, were grown in tanks (12 in. diameter × 6 in. depth; approx. 1000 seedlings/tank) con-

Table 1. *Elution of crude concentrates from charcoal with various solvents*

Solvent	Weight of charcoal (g.)	Volume of solvent (ml.)	Weight of eluate (mg.)	Activity	Percentage recovery
Acetone	100	3 × 100	83	500	3·0
Ether (peroxide-free)-methanol	140	(*a*) ether (100) + methanol (100); (*b*) ether (100) + methanol (30); (*c*) ether (3 × 100)	13·3	800	0·7
Ether (peroxide-free)	250	Continuously extracted for 20 hr.	30	750	1·0
Ethyl acetate-methanol	120	(*a*) ethyl acetate (100) + methanol (30); (*b*) ethyl acetate (100) + methanol (20); (*c*) ethyl acetate (3 × 120)	183	25	8·5
Pyridine	100	5 × 120	712	500	27·4
Chloroform-methanol	70	(*a*) $CHCl_3$ (75) + methanol (30); (*b*) $CHCl_3$ (75) + methanol (20); (*c*) $CHCl_3$ (3 × 100)	79·6	500	4·4
n-Butanol-methanol	100	(*a*) *n*-butanol (90) + methanol (40); (*b*) *n*-butanol (110) + methanol (20); (*c*) *n*-butanol (3 × 100)	150	7	0·2
Acetone-ether (3:1)	440	5 × 500 (mixture stirred during 20–30 min. with each extraction)	178	10,000	100
Acetone-ether (1:1)	100	5 × 120 as in previous extraction	74·9	7500	41

taining distilled water (1·5 l./tank) and carbon (18 g./tank; Sutcliffe and Speakman 'Genster' brand, previously thoroughly washed, first with water and then with a 3:1 acetone-ether mixture). Floats consisting of muslin stretched across closed glass rings carried the seedlings on the surface of the water. It was established that this proportion of water to charcoal gave the maximum yield of crude concentrates and that a reduction in the relative amount of charcoal gave a diminished yield of active material from each tank. 100–120 tanks were set up each week and placed for 5 days in a near-dark room at 25°, and with this technique, after eluting the product from charcoal (see below), some 5–10 mg. of crude concentrates were obtained from each dish and the total weekly production was about 400 mg. of material, probably containing about 2% of the pure germination factor.

Elution of biologically active material from charcoal

The eluting powers of a variety of solvent systems on the wet charcoal were investigated and the results are tabulated below. All evaporations were carried out under reduced pressure (water pump) in an atmosphere of N_2, the temperature of the solution being kept below 35°. The pH of the solutions was maintained at 5·5–6·5 during the evaporations by the cautious addition of $NaHCO_3$ solution and allowance for this has been made in Table 1.

Stability of crude concentrates

A solution of the crude concentrate (activity, 1×10^5) was divided into four fractions and a range of pH values obtained by the addition of 0·01 N-H_2SO_4 or 0·01 N-NaOH respectively (pH values, 2·4, 4·7, 7·0 and 8·0). Each sample was boiled for 10 min. and after cooling, the pH was readjusted to 7·0 and the activity tested. In each case this was found to be $<1{\cdot}0$.

The experiment was repeated with four samples of a solution (activity 5×10^4) adjusted to pH values 1·95, 3·85, 7·0 and 7·95 respectively. These were stored at 4° and the activity values of neutralized portions of the solutions determined after various periods. The results are shown in Table 2.

Table 2. *Variations of activity with time on keeping concentrate solutions of the* Striga *germination factor at different* pH's

Initial pH	Activity values		
	18 hr.	42 hr.	66 hr.
7·95	1	1	1
7·0	250	350	3
3·85	350	8	1
1·95	75	40	1

Preliminary purification of the crude concentrates by solvent extraction

Aqueous solutions of the crude concentrates were extracted with a variety of organic solvents, and the activity of aqueous solutions of the material extracted into the organic phase determined in each case (Table 3).

Examination of the crude concentrates by paper-strip chromatography

The method used was similar to that already described in the case of the *Orobanche* germination stimulant (Brown *et al.* 1951*b*). The chromatograms were run on strips of filter paper (Whatman no. 1; 49 × 17 cm.), using as solvents either the upper layer of a *n*-butanol-acetic acid-water mixture (4:1:5) or *n*-butanol saturated with water. An aqueous solution of the crude material was applied all along the starting line of the chromatogram, the paper was dried at room temperature and then developed with the solvent system. The completed chromatogram after drying was cut crosswise into narrow strips (1 × 17 cm.) which were extracted separately with water (1 ml. per strip) and each extract tested biologically. Typical results are represented graphically in Figs. 1 and 2.

Table 3. *Further purification of crude concentrates of the* Striga *germination factor by solvent extraction*

Volume of original aqueous solution (ml.)	Concentration (mg./ml.)	Activity	Solvent for extraction	Weight of material extracted into organic solvent (mg.)	Activity	Percentage recovery
4·8	6·5	10^4	Ether (6×10 ml.)*	3·6	10^6	>100
5	6·5	10^4	Ethyl acetate (6×10 ml.)	5·0	$0{\cdot}75 \times 10^6$	>100
5	6·5	10^4	$CHCl_3$ (6×10 ml.)	5·8	10^5	>100

* As the aqueous layer still gave a positive biological test, nine ether extractions were used in all subsequent work.

Partial purification of the stimulant by chromatography on cellulose

The columns were prepared by introducing a slurry of cellulose (H. Reeve Angel and Co., London) in water-saturated *n*-butanol into the chromatogram tube ($23 \times 1{\cdot}9$ cm.) and thoroughly washing the cellulose with more solvent before use. The material was brought on to the column, the chromatogram developed and successive fractions of the eluate collected. Each of these was evaporated to dryness below 35° in an atmosphere of N_2 and the residues tested for biological activity. Examples of typical chromatograms are as follows:

(*a*) An aqueous solution (4 ml.; 31·9 mg./ml.; activity 10,000) of an acetone-ether (3:1) eluted crude concentrate was introduced on to a cellulose (23 g.) column, and developed with water-saturated *n*-butanol. After the fractions (total volume approx. 120 ml.) had been collected the column was extruded and extracted with water (150 ml.) and the filtered solution treated in a manner similar to the chromatogram fractions. The activity of each fraction was determined in the usual way and the results are shown in Table 4.

Table 4. *Chromatography of an aqueous solution of a concentrate of the* Striga *germination factor on a column of cellulose*, (*a*)

Fraction no.	Volume of fraction (ml.)	Weight of residue after removal of solvent (mg.)	Activity
1	40	1·7	0·5
2	20	9·2	1×10^6
3	22	3·6	100
4	40	4·1	0
Eluate from extruded column	—	111	0

(*b*) An acetone-ether (3:1) eluted crude concentrate (421 mg.; activity 10,000) after ether extraction yielded a concentrate (23·5 mg.; activity $0{\cdot}75 \times 10^6$) which was dissolved in water (4 ml.) and chromatographed on cellulose (23 g.) as in (*a*) (Table 5).

(*c*) An acetone-ether (3:1) eluted crude concentrate (384 mg.; activity 25,000) after ether extraction yielded a concentrate (13·3 mg.; activity 1×10^6), a portion of which (8·1 mg. in 3 ml.) was chromatographed as above (Table 6).

Table 5. *Chromatography of an aqueous solution of a concentrate of the* Striga *germination factor on a column of cellulose*, (*b*)

Fraction no.	Volume of fraction (ml.)	Weight of residue after removal of solvent (mg.)	Activity ($\times 10^6$)
1	11	4·9	0
2	4	4·1	2·5
3	9	14·7	2·5
4	4	1·5	0·25

Table 6. *Chromatography of an aqueous solution of a concentrate of the* Striga *germination factor on a column of cellulose* (*c*)

Fraction no.	Volume of fraction (ml.)	Weight of residue after removal of solvent (mg.)	Activity
1	40	2·2	0
2	10	4·6	1×10^7
3	12	2·8	2500
4	15	0·6	10

High vacuum distillation of concentrates of the stimulant

(*a*) A concentrate of the *Striga* germination stimulant (26·4 mg.; activity 75,000) was subjected to high vacuum fractional distillation in a micro apparatus. The activities of the various fractions were determined and the results are shown in Table 7.

(*b*) As (*a*). Concentrate (16·4 mg.; activity 1×10^6). (Table 8).

Carbohydrate content of crude concentrates

Solutions of the concentrate (40–60 mg./ml.) were examined for carbohydrates by paper-strip chromatography with the upper layer of a *n*-butanol-acetic acid-water mixture (4:1:5) and using for development the aniline oxalate reagent of Partridge (1950), which distinguishes pentose (pink spots) from hexose (brown spots). As a preliminary to the chromatographic separations, the inorganic material was removed from the solution either by ion-exchange resins or, more simply, by methanol extraction of the solid when the bulk of the salts did not dissolve. The R_F values

Table 7. *High vacuum fractional distillation of a concentrate of the* Striga *germination factor*

Fraction no.	Bath temperature (°)	Pressure ($\times 10^{-4}$ mm.)	Weight of fraction (mg.)	Activity
1	<60	5·5	14·5	0
2	60–115	8·0	5·6	1×10^6
3	115–150	8·0	2·1	Trace
4	150–200	1·4	0·9	0
Residue (methanol-soluble fraction)	—	—	0·5	0

Table 8. *High vacuum fractional distillation of a concentrate of the* Striga *germination factor*

Fraction no.	Bath temperature (°)	Pressure ($\times 10^{-4}$ mm.)	Weight of fraction (mg.)	Activity
1	<60	5	5·4	Trace
2	60–115	7	5·2	1×10^6
3	115–165	3	1·3	Trace
Residue (methanol-soluble fraction)	—	—	1·0	0

obtained in typical chromatograms of the crude concentrate are as follows (figures in parenthesis; starred spots were red, others brown), and they are compared with the values obtained with a series of standard sugars: (*A*) *Striga* concentrate (40 mg./ml.; 0·241*, 0·202, 0·126*, 0·087, 0·036); glucose (0·201); ribose (0·321*); arabinose (0·233*); maltose (0·088); raffinose (0·055). (*B*) *Striga* concentrate (40 mg./ml.; 0·255*, 0·210, 0·144*, 0·105); glucose (0·215); arabinose (0·267*); rhamnose (0·421); xylose (0·298*). (*C*) *Striga* concentrate (60 mg./ml.; 0·207*, 0·162, 0·102*, 0·068, 0·034); maltose (0·071); glucose (0·160); fructose (0·209); arabinose (0·211*); sucrose (0·100); xylose (0·247*); 2-keto-gluconic acid (0·090*). (*D*) *Striga* concentrate (57 mg./ml.; 0·236*, 0·186; 0·123*, 0·100, 0·066); maltose (0·106); galactose (0·164); raffinose (0·059); xyloketose (0·324*).

Amino-acid content of crude concentrates

Solutions of the concentrate (40–60 mg./ml.), after removal of the bulk of the inorganic material as before, were examined for amino-acids by paper-strip chromatography using the *n*-butanol-acetic acid-water mixture as the solvent and ninhydrin as the developing reagent. The details of typical chromatograms are as follows (R_F values in parenthesis as before) and data are provided for standard amino-acids for comparative purposes: (*A*) *Striga* concentrate (60 mg./ml.; 0·613, 0·443, 0·360, 0·280, 0·206, 0·147); norleucine (0·613); valine (0·430); tyrosine (0·352); tryptophane (0·447); glucosamine (0·146). (*B*) *Striga* concentrate (48 mg./ml.; 0·646, 0·443, 0·363, 0·207, 0·146); valine (0·441); aspartic acid (0·168); glutamic acid (0·229). (*C*) *Striga* concentrate (48 mg./ml.; 0·637, 0·459, 0·387, 0·216, 0·137); alanine (0·228); threonine (0·182); norleucine (0·624); valine (0·412); tyrosine (0·347).

SUMMARY

1. Paper chromatographic examinations have demonstrated that the stimulant properties of crude preparations of the *Striga* germination factor may be due to a complex of substances. Using a *n*-butanol-acetic acid-water mixture as solvent, the most active component of the complex is located immediately behind the solvent front and is not a simple sugar or an amino-acid.

2. The purification of concentrates of the germination factor has been partially standarized in that carbon adsorption and elution with acetone-ether mixtures followed by ethereal extraction of the product so obtained, have been adopted as the initial stages.

3. Chromatography on columns of cellulose has been applied to the purification of larger quantities of the concentrates to yield resinous products with high biological activity.

We are greatly indebted to Prof. A. R. Todd, F.R.S., for his advice and interest in this work, to the Colonial Products Research Council for their support of the investigation, and to the Department of Scientific and Industrial Research for a grant to one of us (G.J.T.).

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Reduction of Dehydroascorbic Acid by Bacteria

1. REDUCING MECHANISMS OF *ESCHERICHIA COLI*

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Tkachenko (1936) reported that dehydroascorbic acid (DHA) was reduced to ascorbic acid (AA) in cultures of *Lactobacillus acidophilus*, *L. bulgaricus* and *L. leichmannii*. The system has since been investigated by Esselen & Fuller (1939), Gunsalus & Hand (1941), Stewart & Sharp (1945) and Mapson & Ingram (1951). Esselen & Fuller (1939) investigated eleven strains of *Escherichia coli* and one each of *Aerobacter aerogenes*, *Salmonella pullorum*, *Salm. aertrycke* (*Salm. typhimurium*) and *Salm. enteritidis*. They did not obtain any convincing evidence of reduction except with two strains of *Esch. coli*. Gunsalus & Hand (1941) tested a number of strains of *Esch. coli* isolated from human faeces and found approximately half to be active in reducing DHA. They also found several strains of *Aerobacter cloacae* to be active, but obtained negative results with fifteen strains of streptococci and with baker's yeast. Stewart & Sharp (1945) tested a large number of organisms and found that coliforms and 'coccus forms' were most likely to reduce DHA. Of eighty-eight strains of coliform organisms investigated almost half were active. They finally selected strains of *Esch. coli* and *Staphylococcus albus*. Mapson & Ingram (1951) showed that DHA can be reduced quantitatively to AA by *Esch. coli*, and that this affords a means of estimating DHA.

None of these investigators, however, attempted to account for the distribution of ability to reduce DHA in terms of reducing systems possessed by the bacteria, nor have any investigations been made of the mechanism of DHA reduction. This paper describes an investigation of the mechanism of the reduction which involves, in essence, the addition to the DHA molecule of two atoms of hydrogen.

METHODS

The organism used was a strain of *Esch. coli* (*Bact. coli* Type I 44° negative) isolated in this laboratory and was the same as that used by Mapson & Ingram (1951). It was grown on digest agar (McCartney) in Roux bottles and, except when feeding with formate or lactate, 0·5% (w/v) glucose was incorporated in the medium. The cells were harvested and washed three times with Ringer solution (one-quarter strength) in which they were finally suspended at a concentration of about $7{\cdot}6 \times 10^{10}$ cells/ml. (standardized by opacity tube).

Dry weights were determined by drying 1 ml. of suspension to constant weight over a boiling-water bath. It was found that 1 ml. of suspension containing $7{\cdot}6 \times 10^{10}$ cells had a dry weight of 20 mg. $\pm$ 15% after deducting the weight of the solids of the Ringer solution.

The DHA was prepared by dissolving 200 mg. AA in 50 ml. of glass-distilled water, adding Br_2 in slight excess and shaking. The remaining Br_2 was removed with a stream of air bubbled through the solution.

Experiments on the rate of DHA reduction not involving measurements of hydrogen uptake were carried out as follows. Into a tube, with a two-hole bung carrying a gas inlet reaching to the bottom, were introduced 1 ml. of suspension + 0·5 ml. M-substrate (unless otherwise stated or unless hydrogen were being used as donator) + 2·3 ml. of 0·067 M-phosphate buffer of pH 6·2. The tube was fixed in a water bath at 35° and nitrogen (or hydrogen if used) blown through the mixture. At zero time 0·5 ml. DHA solution (2 mg.) was added. Samples (5 ml.) were removed at 5 min. intervals and, after mixing with an equal volume of 20% (w/v) metaphosphoric acid, were titrated for AA with 2:6-dichlorophenolindophenol.

Investigations involving gas uptakes were carried out using normal manometric (Warburg) technique, the flask contents being titrated for ascorbic acid at the end of the experiments, if necessary.

RESULTS

Specificity of the hydrogen donator. Since the reduction of DHA to AA simply involves the addition of hydrogen, one might assume that a suitable hydrogen-donating system only would be necessary. A number of substrates of some of the dehydrogenases known to be possessed by *Esch. coli* have therefore been tested for their ability to act as hydrogen donators for the DHA-reducing mechanism. Table 1 gives a list of these substances, together with the respective rates of the reaction, with two different suspensions of *Esch. coli*, expressed as hydrogen equivalents (μl.) of DHA reduced/mg. dry wt. of bacteria/hr. ($Q_{H_2}^{DHA}$).

The results show that the DHA-reducing mechanism is not specific towards any single hydrogen donator/dehydrogenase system. There are, however, large differences between the rates of reduction by different systems. Formate, lactate and hydrogen were selected as substrates for use in further work.

Table 1. *Ability of* Esch. coli *to use various substrates as* H-*donators for the reduction of dehydroascorbic acid (DHA)*

(In each case the endogenous reduction has been deducted. $Q_{H_2}^{DHA}$ represents the rate of reduction of DHA expressed as equivalent volumes of H_2 (μl.) transferred from donator to acceptor/mg. dry wt. of bacteria/hr.)

	Suspension 1			Suspension 2		
Substrate	Substrate concn.	pH	$Q_{H_2}^{DHA}$	Substrate concn.	pH	$Q_{H_2}^{DHA}$
—	—	6·2	4	—	—	9·5
Succinate	0·02 M	6·2	15	0·04 M	6·2	9·5
Malate	0·02 M	6·2	9	0·04 M	6·2	13
Lactate	0·02 M	6·2	23	0·02 M	6·2	38·5
Ethanol	2% (v/v)	6·2	12	2% (v/v)	6·2	17·5
Hydrogen	Continuous stream	6·2	25	Continuous stream	7·0	19
Formate	0·02 M	6·2	27	0·02 M	6·2	17
Glucose	0·02 M	6·2	55	0·02 M	7·0	76
Glycine	—	—	—	0·1 M	6·2	32·5

Cell-free enzyme preparations. A cell-free formic dehydrogenase preparation was made by digesting *Esch. coli* cells with trypsin (Stickland, 1929) and a hydrogenase preparation was made by acetone treatment (Bovarnick, 1941). These preparations actively reduced methylene blue, but were completely inactive towards DHA. Mechanical disintegration of the cells, or freeze-drying, also resulted in inactive preparations. The following substances were added in unsuccessful attempts to restore the activity; methylene blue, pyocyanine, glutathione, coenzyme II, yeast extract and boiled and unboiled whole yeast.

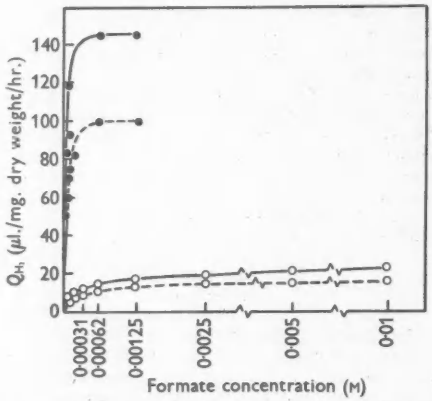

Fig. 1. Effect of formate concentration on rates of reduction of methylene blue and dehydroascorbic acid (DHA) by washed suspensions of *Esch. coli* (—, suspension 1; - - -, suspension 2). ●—●, $Q_{H_2}^{MB}$ (rates of methylene-blue reduction expressed as equivalent volumes of H_2 (μl.) transferred/mg. dry wt./hr.); ○—○, $Q_{H_2}^{DHA}$ (rates of DHA reduction expressed as equivalent volumes of H_2 (μl.) transferred/mg. dry wt./hr.).

Effect of hydrogen-donator concentration on the rate of DHA reduction. In an attempt to throw further light on possible additional links in the chain of reduction reactions, the effects of substrate concentration on the rates of DHA and methylene-blue reduction were next compared. Fig. 1 shows the rates of reduction of DHA and methylene blue for samples of a single suspension, plotted as $Q_{H_2}^{DHA}$ and $Q_{H_2}^{MB}$ against formate concentration; the experiment was later repeated with a different suspension of the same strain of *Esch. coli*.

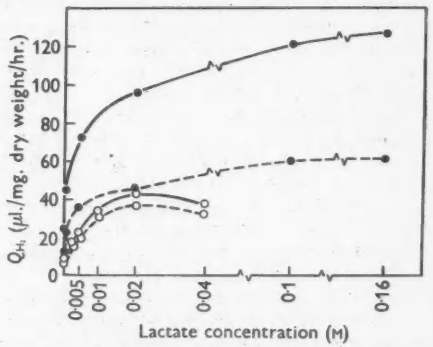

Fig. 2. Effect of lactate concentration on rates of reduction of methylene blue and dehydroascorbic acid (DHA) by washed suspensions of *Esch. coli* (—, suspension 1; - - -, suspension 2). ●—●, $Q_{H_2}^{MB}$ (rates of methylene-blue reduction expressed as equivalent volumes of H_2 (μl.) transferred/mg. dry wt./hr.); ○—○, $Q_{H_2}^{DHA}$ (rates of DHA reduction expressed as equivalent volumes of H_2 (μl.) transferred/mg. dry wt./hr.).

Comparison of the curves shows that in the DHA-reducing system the rate of reaction at any given substrate concentration is far below that for methylene-blue reduction. The K_m for methylene-blue reduction is $3{\cdot}3 \times 10^{-5}$, whereas that for DHA

reduction is $1{\cdot}5 \times 10^{-4}$ approximately (the substrate/rate relation does not quite follow the Michaelis and Menten curve in the latter case). In addition, at formate concentrations above 0·01 M, the rate of DHA reduction decreases with increasing concentration.

Fig. 2 shows the results of similar experiments with lactate as substrate. The lactic dehydrogenase values are calculated from Yudkin (1937) and give a K_m of 5×10^{-3}. The K_m for DHA reduction is (very roughly) 8×10^{-3}.

With lactate, as with formate, the rate of DHA reduction was low compared with that of methylene-blue reduction, but the difference was much greater in one suspension than in the other, although these suspensions had given comparable results in the formate experiments. The inhibitory effect of higher lactate concentrations was more pronounced than with formate.

Effect of increasing the hydrogenase activity. By replacing the glucose in the culture medium with formate or with lactate at the same concentration, the hydrogenase activity of washed suspensions (measured manometrically by the rate of hydrogen uptake with methylene blue as acceptor) was increased by 100 and 250 % respectively. When the rates of DHA reduction by these suspensions using hydrogen as donator were determined, however, it was found that they were much diminished (Table 2).

The results in Table 2 confirm that, with glucose-fed organisms, there is a factor reducing the rate of hydrogen transfer when DHA is the acceptor. The relatively low rates of reduction with formate-fed and lactate-fed organisms further suggest that if some additional factor is required, there is more of it available in cells which are grown on a medium containing glucose.

Table 2. *Effect of lactate and formate feeding on the hydrogenase and dehydroascorbic acid (DHA) reducing activities of* Esch. coli

(The hydrogenase activities ($Q_{H_2}^{MB}$) are given as μl. H_2 taken up/mg. dry wt./hr. $Q_{H_2}^{DHA} = \mu$l. taken up/mg. dry wt./hr. with DHA as acceptor.)

Substrate for cell growth	$Q_{H_2}^{MB}$ (30°)	$Q_{H_2}^{DHA}$ (35°)
Glucose	110	25·3
Formate	220	3·6
Lactate	385	8·6

Quantitative relations between hydrogen used and DHA reduced. Because of the differences between the $Q_{H_2}^{MB}$ and $Q_{H_2}^{DHA}$ values shown in Table 2, experiments were carried out to relate the hydrogen used to the amount of DHA reduced. The hydrogen uptakes were measured manometrically for 25 min., and the flask contents and those of control flasks containing nitrogen were titrated for ascorbic acid. The results are shown in Table 3.

Whereas with glucose-fed organisms the hydrogen uptake is equivalent to the DHA reduction in hydrogen less that in nitrogen, with formate-fed and lactate-fed organisms this is not the case. With

Table 3. *Relation between hydrogen uptake and dehydroascorbic acid (DHA) reduction in suspensions of glucose-fed, formate-fed and lactate-fed* Esch. coli

Substrate for cell growth	Gas	(1) Gas uptake (μl.)	(2) H_2 uptake (1) (H_2) − (1) (N_2) (μl.)	(3) DHA reduction (mg.)	(4) H_2 equiv. of (3) (H_2) (μl.)*	(5) DHA reduction in H_2 (3) (H_2) − (3) (N_2) (mg.)	(6) H_2 equiv. of (5) (μl.)*
Glucose	N_2	−3·0	—	0·084	—	—	—
Glucose	H_2	32·5	35·5	0·354	45·0	0·270	34·5
Formate	N_2	+2·5	—	0·173	—	—	—
Formate	H_2	38·0	35·5	0·341	43·5	0·168	21·5
Lactate	N_2	0·0	—	0·139	—	—	—
Lactate	H_2	47·0	47·0	0·373	47·5	0·234	30·0

* 0·1 mg. DHA is equivalent to 12·8 μl. H_2 at N.T.P.

Table 4. *Suppression of endogenous reduction of dehydroascorbic acid (DHA) by hydrogenase system in washed suspensions of* Esch. coli

($Q_{H_2}^{MB}$ = hydrogenase activity expressed as μl. H_2 taken up/mg. dry wt./hr. with methylene blue as acceptor.)

Substrate for cell growth	(1) Total DHA reduction in N_2 (mg.)	(2) DHA equiv. of H_2 uptake (mg.)	(3) DHA reduction not due to hydrogenase (mg.)	(4) DHA reduction in N_2 (mg.)	(5) (3) as percentage of (4)	(6) Suppression of DHA reduction in N_2 (%)	(7) $Q_{H_2}^{MB}$
Glucose	0·354	0·28	0·074	0·084	88	12	110
Formate	0·341	0·28	0·061	0·173	35	65	220
Lactate	0·373	0·37	0·003	0·139	2	98	385

the latter, the hydrogen uptake is equivalent to the total DHA reduced and with the former, to a figure somewhere between the nitrogen and hydrogen values.

Table 4 suggests that this happens because the hydrogenase system suppresses the endogenous reduction of DHA. Comparison with the respective $Q_{H_2}^{MB}$ values shows that the precentage suppression increases with increasing hydrogenase activity, indicating competition between the two reducing systems. Thus in lactate-fed organisms, with a high $Q_{H_2}^{MB}$, the endogenous system is wholly suppressed, and all the reduction is at the expense of the hydrogen/hydrogenase system, while in glucose-fed organisms the reduction by the endogenous system must first be deducted in order to obtain equivalence.

Table 5. *Rates of dehydroascorbic acid (DHA) reduction in the presence of one or more hydrogen donators expressed as equivalent volumes of* H_2 *(µl.) transferred from donator to acceptor/mg. dry wt./hr.* ($Q_{H_2}^{DHA}$)

Hydrogen donator	$Q_{H_2}^{DHA}$
(1) None	15
(2) 0·01 M-Formate	46·5
(3) 0·02 M-Lactate	52
(4) (2) + (3)	63
(5) Hydrogen (continuous stream)	30·5
(6) (2) + (5)	45
(7) (3) + (5)	59
(8) (2) + (3) + (5)	62
(9) 0·02 M-Glucose	84

Effect of mixed substrates. Table 5 shows the effect on the rate of DHA reduction of the presence of more than one dehydrogenase substrate, each at its optimal concentration (cf. Figs. 1 and 2) or, as is the case with hydrogen, at saturation. The rates of DHA reduction are not additive, but appear to have a maximum $Q_{H_2}^{DHA}$ of 63, except in the case of glucose.

DISCUSSION

As already stated, the results suggest that the mechanism of reduction of DHA by *Esch. coli* is not very specific with regard to the hydrogen-donating mechanism, since any of several dehydrogenases possessed by the organism which were tested, and hydrogenase itself, can serve to effect the reduction; also the rates of reduction vary considerably with the different hydrogen-donators and also from one suspension to another. This may be due, at least in part, to the differing amounts of enzymes present and to their various pH and substrate-concentration relationships. Among the dehydrogenases tested are representatives of the aerobic (glycine), cytochrome-linked (succinic, lactic and formic) and coenzyme-linked (malic, ethanol and glucose) types.

The bulk of the evidence presented, however, suggests that the hydrogen-donating mechanism does not constitute the whole system, as is the case when methylene blue is reduced, but that one or, more probably, several factors are involved, and that different treatments may result in the loss of one or other of these factors.

The work comparing the effect of hydrogen-donator concentration on the rates of DHA and methylene-blue reduction, and that using mixed substrates, has shown that the rate of DHA reduction is limited, but not by the potential rate of hydrogen transfer. When glucose is added as hydrogen donator, the rate of reduction is higher than that which is achieved by the combined effects of formate, lactate and hydrogen. It may be that there are two mechanisms for DHA reduction and that for one of these only glucose can act as hydrogen donator.

The fact that cell-free dehydrogenase and hydrogenase preparations are active towards methylene blue but not towards DHA shows that in this milieu DHA is not spontaneously reducible and suggests that a DHA-activating enzyme may be necessary before reduction can take place. Further evidence for a DHA-activating enzyme is the fact that although D-*iso*dehydroascorbic acid and the oxidized forms of hydroxytetronic and reductic acids are reduced, the rates of reduction are much slower than that of DHA (Mapson & Ingram, 1951). The experiments with formate-fed and lactate-fed cells indicate that a factor is required which is best produced if the organisms are fed on glucose. Since the yield of cells is greater on formate or lactate than on glucose, it may be suggested that the missing factor in this case is not of great importance in general metabolism and is, therefore, probably not one of the recognized hydrogen carriers.

Previous work on coupled oxidations and reductions has, however, shown that a hydrogen carrier is necessary before the reactions will take place *in vitro* (Green, Stickland & Tarr, 1934) and certain aspects of the present work, such as the failure of acetone powders and mechanically disintegrated preparations of *Esch. coli* to effect the reduction, suggest that a hydrogen carrier may be essential in this reaction. Further experiments have indicated that this is the case, and work is in progress to substantiate these indications.

SUMMARY

1. Hydrogenase and formic, lactic, succinic, malic, ethanol, glucose and glycine dehydrogenases, with their substrates, can act as hydrogen-donating mechanisms for dehydroascorbic acid (DHA) reduction by *Escherichia coli*.

2. Cell-free dehydrogenase preparations will not reduce DHA, indicating that some essential factor or factors is absent from them. When absent, the

factor is not replaceable by methylene blue, pyocyanine, glutathione, coenzyme II, yeast extract, or boiled or unboiled whole yeast.

3. The effect of hydrogen-donator concentration on the rate of DHA reduction, as compared with the rate of methylene-blue reduction, indicates that the rate of hydrogen transfer may be greatly reduced in the DHA-reducing system.

4. Growth on lactate or formate in place of glucose increases the hydrogenase activity, but decreases the DHA-reducing activity.

5. When more than one hydrogen donator is present, the rates of DHA reduction are not additive. The rate of reduction when formate, lactate and hydrogen are present together is not as great as when glucose is present alone.

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Oxidation of Phenylacetic Acid by *Penicillium chrysogenum*

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Phenylacetic acid (PA) has been shown by Singh & Johnson (1948) to be almost wholly consumed by *Penicillium chrysogenum* in fermentations producing penicillin. Only a small fraction entered the penicillin produced. The present study has indicated that the rest is wholly or partly oxidized, beginning with the acetic acid residue.

This type of oxidation in moulds has not hitherto been described. Supniewski (1942) has, however, claimed that his 'Bacille pyocyanique' was capable of oxidizing PA by way of benzoic acid.

EXPERIMENTAL

Methods

(1) *Determination of phenylacetic acid.* As acetic acid was a normal contaminant of the fluids examined, it was necessary to steam-distil them before extraction with ether, following the procedure described by Friedemann (1938), but using Hockenhull's (1949) modification of the apparatus. Results had a precision of $\pm 5\%$. Benzoic acid interfered because it behaved in almost the same way as PA.

(2) *Growth conditions.* The material for use in these studies was obtained by growing the mould upon the synthetic medium of Jarvis & Johnson (1947). (Lactose, 22·5; glucose, 7·5; ammonium acetate, 3; PA, 2; KH_2PO_4, 3; $MgSO_4.7H_2O$, 0·25; $FeSO_4.7H_2O$, 0·10; $CuSO_4.7H_2O$, 0·004; $MnSO_4.4H_2O$, 0·004; Na_2SO_4, 0·5; $CaCO_3$, 10 g./l. distilled water.) The medium was adjusted to pH 6·3 with 10N-NaOH before sterilization for 15 min. at 15 lb./sq.in. It was distributed in 60 ml. quantities into 250 ml. conical flasks. The flasks were each inoculated with 3% of a vegetative inoculum and incubated at 25° on a rotary shaker at 180 rev./min.

The inoculum was prepared as follows: A suspension of 10^7 to 10^8 spores/ml. in 0·1% aqueous Calsolene oil HS (Imperial Chemical Industries Ltd.) was made up from a 6- to 8-day culture on glycerol-molasses-peptone agar of the organism which had been derived from *P. chrysogenum* Q176 by ultraviolet light mutation. This suspension (3 ml.) was inoculated into 60 ml. of a development medium (containing corn steep liquor (C.S.L.), brown sugar and 0·1% PA) in a 250 ml. conical flask. This was incubated for 40 hr. at 25° on a rotary shaker at 180 rev./min.

(3) *Respiration of mould suspensions in presence of PA.* The preparations used for respirometric work with Warburg apparatus were made as follows. Mycelium from the 60 ml. culture described above was centrifuged, washed in 60 ml. 0·05M-sodium phosphate buffer, pH 7·2, centrifuged and again suspended in 60 ml. buffer in a 250 ml. conical flask. Four 6 mm. glass beads were added and the flask was shaken at 25° for 16 hr. to reduce O_2 consumption in the absence of substrate. The felt was again centrifuged off and finally resuspended in a further 60 ml. of buffer. In the presence of PA this suspension gave an increase in respiration of 50–200% over the blank.

The Warburg vessels usually contained 2 ml. suspension (20–30 mg. dry wt.). The standard substrate concentration

was 0·01 M. The Q_{O_2} (PA) (μl. O_2 absorbed/mg. dry wt. mycelium/hr.) was found by the usual method; Q_{O_2} (PA) = Q_{O_2} (total)-Q_{O_2} (blank). In presence of 0·01 M-substrate the rate of O_2 uptake was almost constant for at least 1 hr.

(4) *Disappearance of PA in presence of the mould.* A grown culture was harvested, filtered, washed and resuspended in pH 7·2 0·05 M-sodium phosphate buffer.

PA was added at about 2·5 mg./ml. Tests were usually carried out in a volume of 60 ml. in 250 ml. conical flasks at 25° on the shaker (180 rev./min.) for a period of 16 hr. About 30–40 % of the added PA was utilized at a substantially constant rate.

RESULTS

Examination of PA metabolism in typical fermentations

Standard fermentations were set up on Jarvis & Johnson (1947) medium (60 ml. in each of twenty-four 250 ml. flasks). At intervals up to 100 hr. pairs of flasks were withdrawn, the contents were washed and starved suspensions prepared (§3 of Methods). The filtrates were analysed for PA. The activity of the suspension was tested against PA and also against the broth filtrate at its original strength (whole broth).

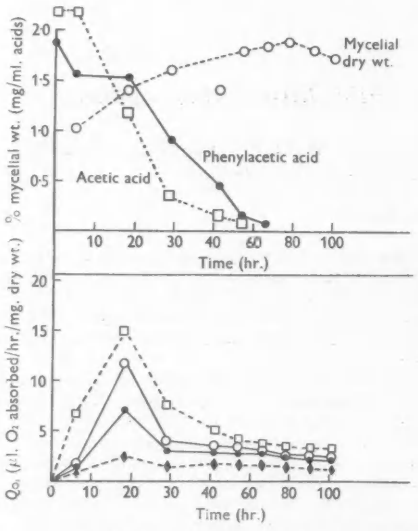


Fig. 1. Relationship between enzymic activity, growth and medium constituents in shaken cultures of *P. chrysogenum* Q 176 at 25°. Graphs marked PA and HPA represent total Q_{O_2} in presence of PA and *p*-hydroxyphenylacetic acid respectively. □—□, whole broth; ○—○, HPA; ●—●, PA; ◆- - -◆, blank.

Fig. 1 shows that the respiratory activity against PA and broth constituents was greatest after about 18 hr. By this time the PA level had begun to fall rapidly. The mycelial weight was then about 75 % of its maximum.

Accordingly, preparations for further study were harvested at 16–18 hr.

Relation of Q_{O_2} (PA) to substrate concentration

Respirometer experiments were carried out using different levels of PA (Table 1).

Table 1. *Relation of Q_{O_2} (PA) to substrate concentration*

(Incubation mixture: washed starved suspension prepared as described in section 3 of Methods, 2 ml. (25–30 mg. dry wt.); substrate, 0·2 ml. in side bulb.)

	PA (Na salt) (M)	Q_{O_2} (PA) 0–20 min.
Exp. 1	0·00640	3·90
	0·00250	3·55
	0·00100	3·45
	0·00040	3·45
	0·00016	3·01
	0·000064	3·12
	0·000026	2·55
(Endogenous respiration)		(2·45) Q_{O_2} (blank)
Exp. 2	0·100	2·93
	0·04	3·00
	0·016	4·36
	0·0064	4·10
	0·0026	4·21
	0·0010	3·98
	0·0004	3·88
	0·00016	3·48
(Endogenous respiration)		(1·62) Q_{O_2} (blank)

At the lowest concentrations employed there was a decrease in Q_{O_2} during the 20 min. reaction time, which, however, could be mainly accounted for by exhaustion of substrate. The differences during the 5 min. between readings were too small, compared with the inherent error of the method, to show whether any change in respiration was associated with change in substrate concentration. At this lowest level the oxygen consumption for total oxidation was calculated and found to be 8·6 mol. oxygen/mol. PA as against a theoretical value of 9·0 mol./mol. for complete conversion of PA to carbon dioxide.

At 0·000064 M and above, the respiratory rate was almost constant except at the very high (0·10 and 0·04 M) concentrations where there was a decline, possibly due to toxicity.

Relation of enzyme activity to pH *value*

Respirometer experiments were carried out in 0·05 M-sodium phosphate buffers from pH 5·6 to 8·4. Controls without substrate were carried out at each pH value. The Q_{O_2} (PA) was found to increase towards the highest pH value (Fig. 2).

Accelerated reduction of methylene blue in presence of PA

A response to PA was obtained when the mould was incubated anaerobically with methylene blue in Thunberg tubes. For this, 5 ml. of washed, starved suspension (about 25 mg. dry wt./ml.) were employed in the main tube. The side bulbs each contained 1·0 ml. of 0·1% methylene blue with or without 1·0 ml. of 0·1 M-PA. Incubation was carried out at 26°. Decolorization times were: blank, 10·5 min.; with substrate, 7·5 min. (means of four values).

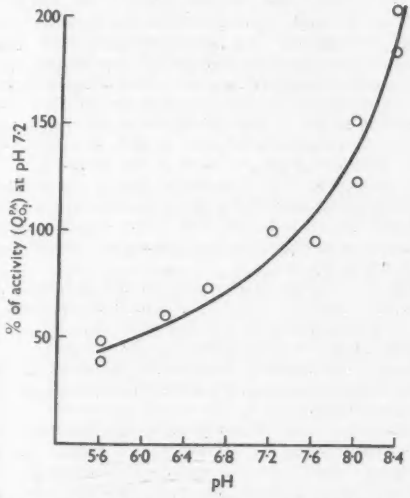


Fig. 2. Relationship between pH and PA oxidation by washed suspensions of *P. chrysogenum* Q176 at 26°.

Substrate specificity of the oxidizing systems

Under standard conditions with the Warburg respirometer the following substrates showed marked enhancement of respiration over the blanks: hydrocinnamic, benzoylformic, DL-hydratropic and *p*-hydroxyphenylacetic acids. Smaller increases were shown with benzoic, DL-mandelic, D-hydratropic acids. The responses of β-phenyl-*iso*butyric and *p*-hydroxybenzoic acids were negligible (Table 2).

It must be emphasized that the above results were obtained from different preparations and should, therefore, be interpreted only in a qualitative sense.

Inhibitor studies

Effects on respiration. The effects of various compounds (brought to pH 7·2 with sodium hydroxide or hydrochloric acid) at 0·0001 M were tested upon the standard respiration systems (0·01 M-PA). The substances tested were chosen either for structural similarity to PA or because they had been shown to have inhibitory activity against special types of enzymes. The following substances were tested: *p*-hydroxyphenylacetic, *p*-bromophenylacetic, *p*-aminophenylacetic, *p*-nitrophenylacetic, DL-α-chlorophenylacetic, DL-α-aminophenylacetic, D or DL-α-methylphenylacetic (hydratropic), αα-dimethylphenylacetic (β-phenyl*iso*butyric), benzoic, *p*-hydroxybenzoic, *o*-hydroxybenzoic, *o*-aminobenzoic, *p*-aminobenzoic, *p*-methoxybenzoic, *o*-chlorobenzoic, *m*-chlorobenzoic, *p*-chlorobenzoic, *p*-bromobenzoic, *p*-iodobenzoic, *p*-nitrobenzoic, benzenesulphonic, toluene-α-sulphonic, toluene-*p*-sulphonic, naphthalene-2-sulphonic, hexahydrophenylacetic, 2-naphthylacetic, cinnamic, hydrocinnamic, DL-mandelic, monochloroacetic, trichloroacetic, monoiodoacetic and dichlorophenoxyacetic acids, phenylethylamine, phenylacetamide, benzylcyanide, *N*-phenylacetyl-α-alanine, *N*-phenylacetyl-β-alanine, *p*-fluorophenylethylamine, sulphanilamide, α-alanine, β-alanine, phenylalanine, *p*-cresol, a commercial mixture of *o*-, *m*- and *p*-cresols, *p*-nitrophenol, 2:4-dinitrophenol, sodium azide, hydroxylamine, sodium fluoride, mercuric chloride, copper sulphate, sodium arsenite, sodium arsenate, quinine, acriflavine, methylene blue, semicarbazide, 2:3-dimer-

Table 2. *Specificity of the oxidizing systems*

(Incubation mixture: suspension as § 3 of Methods, 2 ml.; substrate, 0·2 ml. of 0·1 M-Na salt. Each figure is the mean of two readings not differing from each other by more than 0·10. The respiration of the system in presence of PA is given each time as this varied from preparation to preparation, as these had to be prepared afresh for each experimental run.)

Substrate (*S*) 0·01 M	Q_{O_2} (*S*)	Q_{O_2} (control)	Q_{O_2} (no substrate)
Benzoic acid	0·25	2·34	3·35
	1·30	4·89	2·40
DL-Mandelic acid	0·24	1·30	3·21
Hydrocinnamic acid	1·16	1·17	1·93
D-Hydratropic acid	0·39	1·17	1·93
DL-Hydratropic acid	1·00	1·30	3·21
β-Phenyl*iso*butyric acid	0·15	1·3	5·40
p-Hydroxybenzoic acid	0·12	2·34	3·35
p-Hydroxyphenylacetic acid	9·19	4·89	2·40
Benzoylformic acid	0·62	1·09	2·38

captopropanol (BAL), dithizone, 8-hydroxyquinoline and salicylaldoxime. The only significant inhibitions (per cent residual activity given in parentheses) were produced by *p*-fluorophenylethylamine (60), cinnamate (69), fluoride (65), iodoacetate (23) and mercuric chloride (0).

Effects of disappearance of PA. Investigations were also carried out by the acidimetric technique described in § 1 of Methods. Owing to the inability of the analytical techniques to distinguish between PA and benzoic acid, blockage of benzoic acid oxidation with consequent accumulation would be indistinguishable from failure to bring about the initial oxidation of PA. The compounds listed above were employed at 0·0015 M. Inhibitions (per cent residual activity given in parentheses) were obtained with *p*-fluorophenylethylamine (51), sodium cinnamate (47), sodium iodoacetate (51), mercuric chloride (48), *p*-cresol (47), 'mixed cresols' (4), sodium azide (8) and hydroxylamine (8). The last four had no effect on the oxygen uptake due to PA. The effect of azide and hydroxylamine may indicate the participation of a metallo-enzyme in the system, possibly at the benzoic acid level.

Arsenite, quinine, acriflavine and thiocyanate were ineffective. Potassium cyanide was itself destroyed by the system. Copper sulphate was ineffective even up to 0·03 M. All results were corrected for the effect of inhibitors upon the blanks.

Isolation of intermediate metabolites

A mixture containing 5 mg./ml. PA in 500 ml. of washed suspension was allowed to react according to the technique described above. It was thought that phenolic bodies might arise by oxidation of the ring in a manner analogous with the conversion of phenylalanine to tyrosine in mammals (Jervis, 1947). Filtered samples were therefore tested for phenols, either by coupling with diazotized sulphanilic acid (method of Fox, 1922) or by coupling with 2-chloro-4-nitrophenyldiazonium naphthalene-2-sulphonate (by the methods described by Heinrich & Schuler, 1947, 1948). Red-brown or red-purple colours were obtained, respectively. The chromogenic product seemed, however, to pass into ether from neutral or even mildly alkaline aqueous solution, and no 'acidic' product could be isolated. No fraction corresponding to *p*-hydroxyphenylacetic acid could be isolated, although this was not surprising in view of its ready oxidizability (usually thrice that of PA).

Attempts to trap quinones by the addition of aniline to the reaction mixture were also unsuccessful, probably because of a general toxic effect on the mould. The results of inhibitor and other studies indicated that side-chain oxidation was probable. If this were so, the formation of benzaldehyde might be expected and further efforts were directed to its isolation. The addition of phenylhydrazine oxalate (1 mg./ml.) to the reaction mixture completely inhibited PA oxidation. Sulphite, however, proved non-inhibitory and gave the following results:

Exp. 1. *Production of benzaldehyde from PA.* Felt from 500 ml. of medium was filtered and resuspended in 500 ml. of solution containing 10 g. KH_2PO_4, 25 g. Na_2SO_3 and 2·5 g. PA (Na salt). The mixture was shaken at 25° for 47 hr. After 22 hr. a smell of benzaldehyde was detected.

The filtered broth (about 400 ml.) was made blue with thymolphthalein by adding 10% (w/v) Na_2CO_3 and extracted with three 120 ml. portions of ether. After washing with water, the ether extract was concentrated under reduced pressure. The aqueous residue (2–3 ml.) was diluted to 10 ml. with ethanol and divided into two portions. From these, benzaldehyde 2:4-dinitrophenylhydrazone (yield 65 mg., m.p.: isolated material 240–241°) and benzaldehyde semicarbazone (yield 70 mg., m.p.: isolated material 216–218°) were prepared; melting points of the authentic derivatives and mixed melting points were identical with these values. (Semicarbazone: Found: C, 58·3; H, 5·4; N, 25·8, 26·0. Calc. for $C_8H_9ON_3$: C, 58·9; H, 5·6; N, 25·8%.)

Exp. 2. *Production of benzaldehyde from* DL-*mandelic acid.* Exp. 2 differed from Exp. 1 in that 2·5 g. of DL-mandelic acid replaced the 2·5 g. of PA. The whole ethanolic extract prepared as in Exp. 1 yielded 108 mg. of semicarbazone (m.p. 210–216°).

Exp. 3. *Production of benzaldehyde from benzyl alcohol.* An experiment similar to Exp. 2 was carried out with 2·5 g. of benzyl alcohol instead of PA. The whole ethanolic extract yielded 242 mg. of semicarbazone (m.p.: 210–216°).

Exp. 4. *Production of acetophenone from* DL-*hydratropic acid.* Exp. 1 was repeated with 2·5 g. DL-hydratropic acid instead of the PA.

After 1 hr. a smell of acetophenone was apparent. After 23 hr. the mixture was extracted as in Exp. 1. All the alcoholic extract was used to make a semicarbazone. Yield 110 mg.; m.p. isolated material, 190–196°; authentic acetophenone semicarbazone, 198–199°; mixed, m.p. 193–197°; benzaldehyde semicarbazone, 216–218°. (Found: C, 60·6; H, 6·2; N, 23·4; Calc. for $C_9H_{11}ON_3$: C, 61·0; H, 6·3; N, 23·7%.)

Later experiments showed that sulphite was necessary for the production of acetophenone.

Exp. 5. Exp. 1 was repeated with 2·5 g. benzoylformic acid instead of PA. Although a slight smell of benzaldehyde appeared on making the solution alkaline, no derivative could be isolated by the above technique.

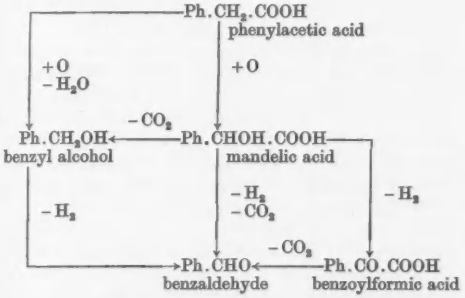
Nature of penicillins produced on synthetic medium

A complete fermentation was carried out on a 60 ml. scale by the method described above. The broth filtrate was assayed at 6 days for the various penicillins (paper chromatography). The relative percentage activities of the various components was as follows: (*Staphylococcus aureus* units): *p*-hydroxybenzyl (*X*) 0·1; X_1, 0·9, benzyl (*G*) 63·4; amyl, 5·6; *n*-heptyl (*K*), 23·7; K_1, 0·2; pentenyl (*F*), 6·1%. This extremely low proportion of penicillin *X* indicated the formation of no substantial quantity of *p*-hydroxyphenylacetic acid from PA.

DISCUSSION

The isolation of benzaldehyde has shown that the initial oxidation of PA chiefly takes place in the side chain, although ring oxidation cannot be completely excluded. The methylene-blue technique indicated that the first oxidative attack on the side chain was probably a dehydrogenation.

Possible routes to benzaldehyde might be as follows:



Since benzyl alcohol and mandelic acid have both given rise to benzaldehyde, neither can be excluded as an intermediate. Benzoylformic acid, however, although oxidized by the system, has not been found to give rise to significant quantities of benzaldehyde and is thus unlikely to be an intermediate. The finding that hydratropic acid may be converted to acetophenone indicates the possibility of a homologous reaction for PA (without prior oxidation to the keto acid). The above is in contrast to the finding of Gunsalus, as reported by Stanier (1950), that benzoylformic acid is an oxidation product of mandelate and may be further transformed to benzaldehyde by *Pseudomonas fluorescens*.

The possibility of direct oxidation of PA to benzaldehyde must not be ignored, although such reactions ('perhydroxylations') generally require molecular oxygen or peroxide.

The transformation of benzaldehyde to benzoic acid, whether spontaneous or enzymically catalysed, is presumed to be the next stage in PA degradation. Possible mechanisms for further oxidation might resemble those discussed in the reviews by Happold (1950) and Stanier (1950).

SUMMARY

1. Phenylacetic acid is oxidized completely to carbon dioxide by mycelial suspensions of a mutant of *Penicillium chrysogenum* Q 176.

2. The oxidative activity of such suspensions was greatest when the parent culture had achieved three-quarters of its maximum growth, the total respiratory activity being highest at this time.

3. Respiration in presence of phenylacetic acid increased over the pH range 5·6–8·4.

4. Benzaldehyde has been characterized as a product of PA oxidation.

5. Similar suspensions oxidized benzoic, hydrocinnamic, DL-mandelic, DL- and D-hydratropic, *p*-hydroxyphenylacetic, *p*-hydroxybenzoic and benzylformic acids and benzyl alcohol, but not β-phenyl*iso*butyric acid. Benzaldehyde has been isolated after biological oxidation of DL-mandelic acid or benzyl alcohol: acetophenone has been obtained by oxidation of hydratropic acid. Benzoylformic acid does not yield benzaldehyde in the presence of the mould.

6. The oxidation of phenylacetic acid is inhibited by mercuric or iodoacetate ions.

We wish to thank Dr B. A. Hems and Dr J. Elks for providing many of the compounds tested as inhibitors. We acknowledge also the technical assistance of Mr D. Goodinson and Miss E. Theakston.

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Studies in the Biochemistry of Micro-organisms

85. CYCLOPOLIC AND CYCLOPALDIC ACIDS, METABOLIC PRODUCTS OF *PENICILLIUM CYCLOPIUM* WESTLING

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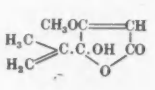
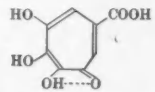
The genus *Penicillium* has been separated by Raper & Thom (1949) in their *Manual of the Penicillia* into four major sections, Monoverticillata, Asymmetrica, Biverticillata-Symmetrica and Polyverticillata. The section Asymmetrica is further divided into five subsections: Divaricata, Velutina, Lanata, Funiculosa and Fasciculata. The subsection Asymmetrica-Fasciculata, in its turn, is subdivided into ten series, one of which, the *P. cyclopium* series, contains four accepted species and one variety, i.e. *P. cyclopium* Westling, *P. cyclopium* West. var. *echinulatum* Raper & Thom, *P. puberulum* Bainier, *P. martensii* Biourge, and *P. aurantio-virens* Biourge.

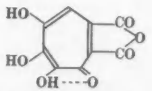
The *P. cyclopium* series has proved to be of considerable biochemical interest. In 1913, Alsberg & Black isolated from laboratory cultures of *P. puberulum* a metabolic product which they named penicillic acid. Birkinshaw, Oxford & Raistrick (1936) isolated from Westling's type strain of *P. cyclopium* considerably larger yields of penicillic acid and established its molecular structure (structure I). Birkinshaw & Raistrick (1932), working with Alsberg & Black's strain of *P. puberulum*, showed that, after almost 20 years in laboratory culture, this strain now gave much smaller yields of penicillic acid than were reported by Alsberg & Black. In addition, however, two new metabolic products, puberulic and puberulonic acids, were isolated. The same two acids were also isolated by Birkinshaw & Raistrick (1932) from *P. aurantio-virens* and by Oxford, Raistrick & Smith (1942*b*) from *P. johannioli* Zaleski (= *P. martensii* Biourge). Penicillic acid, puberulic acid and puberulonic acid have significant antibacterial activity, particularly against Gram-positive bacteria (Oxford, Raistrick & Smith, 1942*a*, *b*). Birkinshaw & Raistrick (1932) and Barger & Dorrer (1934) prepared a number of derivatives and breakdown products of puberulic and puberulonic acids but were unable to advance plausible structural formulae for them. Dewar (1945), in a novel attempt to interpret this experimental evidence and additional evidence on stipitatic acid (Birkinshaw, Chambers & Raistrick, 1942), suggested that puberulic acid might contain a seven-carbon ring and be in fact a derivative of what he proposed to call tropolone (*cyclo*heptatrienolone or hydroxy-*cyclo*heptatrienone), at that time unknown, puberulic acid thus becoming a dihydroxytropolonecarboxylic acid of undetermined orientation. Corbett, Johnson & Todd (1950*a*) have confirmed this view and have shown that puberulic acid has structure (II). The structure of puberulonic acid, which on heating with dilute sulphuric acid loses a molecule of carbon dioxide and is converted into puberulic acid (Corbett, Hassall, Johnson & Todd, 1950), seems now to be satisfactorily settled. Three possible structural formulae were advanced by Corbett *et al.* (1950*a*), but doubt was thrown on these formulae by Aulin-Erdtman (1950) who prefers structure (III) (see also Cook & Loudon, 1951). This structure has been confirmed by Aulin-Erdtman & Theorell (1950) and has now been accepted by Johnson, Sheppard & Todd (1951).



(I) Penicillic acid



(II) Puberulic acid



(III) Puberulonic acid

Other naturally occurring substances which have been shown to contain the tropolone nucleus are stipitatic acid from *P. stipitatum* Thom (Birkinshaw, *et al.* 1942; Corbett, Johnson & Todd, 1950*b*), the α-, β- and γ-thujaplicins from *Thuja plicata*, the western red cedar tree (Erdtman & Gripenberg, 1948*a*, *b*), colchicine (Dewar, 1945; Lettré, 1947; Arnstein, Tarbell, Huang & Scott, 1948) and purpurogallin which occurs as the glycoside eriophyesin (Barltrop & Nicholson, 1948; Haworth, Moore & Pauson, 1948).

We have examined a number of strains of *P. cyclopium*, a species which is well defined morphologically, but many of the strains of this ubiquitous species have shown no biochemical features of interest. We have, however, obtained from each of two strains, recently isolated from natural sources, two hitherto undescribed mould metabolic products for which we propose the names cyclopolic acid and cyclopaldic acid, and it is the purpose of the present communication to describe these substances. The two strains of *P. cyclopium* used, while indubitably strains of *P. cyclopium* Westling morphologically, are quite different in appearance. Strain no. 77 is a typical green sporing strain. Strain no. 85, on the other hand, while morphologically similar to no. 77 in all other respects, differs from it in its total lack of green conidial pigment. It is almost pure white in colour and has been described by our colleague, Mr G. Smith (see Smith, 1951), as a new variety, *P. cyclopium* Westling var. *album* G. Smith.

CYCLOPOLIC ACID

Cyclopolic acid, $C_{11}H_{12}O_6$, is closely related, structurally, to cyclopaldic acid, $C_{11}H_{10}O_6$, as is indicated *inter alia* by the fact that when cyclopolic acid is oxidized with potassium periodate in hot dilute sulphuric acid solution cyclopaldic acid is formed in fairly good yield. For this reason, and also because cyclopolic acid is formed by both strains nos. 85 and 77 in much greater yields than is cyclopaldic acid, most of the experimental work on the chemistry and structure of these two acids was carried out on cyclopolic acid.

Cyclopolic acid, $C_{11}H_{12}O_6$, forms colourless plates, m.p. 147–148° (decomp.). It has no optical activity. It contains one methoxyl group and one methyl group attached to carbon. It dissolves at once, with liberation of carbon dioxide, in aqueous sodium bicarbonate and forms a colourless solution both in this alkali and in aqueous sodium hydroxide. It titrates conductometrically as a dibasic acid. An aqueous solution of cyclopolic acid gives an intense stable purple colour, almost indistinguishable from that given by salicylic acid, with aqueous ferric chloride; immediately decolorizes bromine water with the formation of a precipitate soluble in excess of the reagent; quickly gives a deep-yellow crystalline precipitate with Brady's reagent (0·32% 2:4-dinitrophenylhydrazine in 2N-hydrochloric acid); gives a negative reaction with Schiff's reagent; reduces ammoniacal silver nitrate solution on warming.

The following functional derivatives have been prepared: cyclopolic acid mono-2:4-dinitrophenylhydrazone, $C_{11}H_{12}O_5{:}N.NH.C_6H_3(NO_2)_2$, deep-yellow microprisms, m.p. 190·5–191° (decomp.); cyclopolic acid thiosemicarbazone, $C_{11}H_{12}O_5{:}N.NH.CS.NH_2$, colourless microprisms, m.p. 180·5–182° (decomp.), which are readily soluble in cold aqueous sodium bicarbonate and give in ethanolic solution an orange colour with ethanolic ferric chloride; methyl cyclopolate monomethyl ether, $C_{10}H_{10}O_3(OCH_3).(COOCH_3)$, m.p. 87·5–88°, by treatment of cyclopolic acid with ethereal diazomethane. It gives no colour with aqueous ferric chloride, but quickly gives a yellow precipitate with Brady's reagent. Cyclopolic acid monomethyl ether mono-2:4-dinitrophenylhydrazone, $C_{12}H_{14}O_5{:}N.NH.C_6H_3(NO_2)_2$, yellow needles, m.p. 287° (decomp.), is soluble in cold saturated aqueous sodium bicarbonate.

When cyclopolic acid is heated above its melting point, or when a solution of cyclopolic acid in 2N-sulphuric acid is boiled, the acid is converted, with the loss of one molecule of water, into a colourless crystalline compound, cyclopolide, $C_{11}H_{10}O_5$, m.p. 169°. On acetylation, either with acetic anhydride and anhydrous sodium acetate or with acetic anhydride and pyridine, cyclopolic acid gives, in good yield, a triacetate, $C_{11}H_9O_3(O.CO.CH_3)_3$, m.p. 117°, which, unlike the parent substance, does not dissolve in cold 2N-sodium hydroxide, gives no colour with ethanolic ferric chloride nor any precipitate with Brady's reagent. This triacetate, called for convenience cyclopolide triacetate, on hydrolysis with boiling 2N-sulphuric acid, gives three molecules of acetic acid and an almost theoretical yield of cyclopolide. Cyclopolide is readily soluble in cold aqueous sodium bicarbonate, gives a pale-yellow solution in cold 2N-sodium hydroxide, a deep ruby-red colour very similar to that given by cyclopaldic acid, with ethanolic ferric chloride and an immediate orange precipitate with Brady's reagent. The following derivatives of cyclopolide have been prepared: cyclopolide monomethyl ether, $C_{11}H_9O_4(OCH_3)$, m.p. 169–170°, insoluble in cold aqueous 2N-sodium hydroxide and giving no colour with ethanolic ferric chloride; mono-2:4-dinitrophenylhydrazone of cyclopolide monomethyl ether, $C_{12}H_{12}O_4{:}N.NH.C_6H_3(NO_2)_2$, deep-orange needles which have no sharp melting point; diacetate of cyclopolide monomethyl ether, $C_{12}H_{12}O_4(O.CO.CH_3)_2$, m.p. 133·5–134°, which, unlike cyclopolide monomethyl ether, no longer gives a precipitate with Brady's reagent.

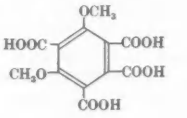
The experimental evidence presented so far justifies the working hypothesis that there are present in the molecule of cyclopolic acid the following groups: one OCH_3 group actually estimated; one C-CH_3 group actually estimated in both cyclopolic acid and cyclopolide; one OH group to account for the intense purple ferric chloride colour given by cyclopolic acid and the absence of this colour in methyl cyclopolate monomethyl ether; one carbonyl group, probably a CHO group, explaining the formation of cyclopolic acid thiosemicarbazone

and mono-2:4-dinitrophenylhydrazone; one COOH group indicated by conductometric titration, which accounts for the liberation by cyclopolic acid of carbon dioxide from aqueous sodium bicarbonate and which is esterified in methyl cyclopolate monomethyl ether; one CH_2OH group, vicinal to the COOH group, to account for the formation of cyclopolide with the loss of one molecule of water. On this hypothesis the formula for cyclopolic acid may be written $C_6.(OCH_3).(CH_3).(OH).(CHO).(COOH).(CH_2OH) = C_{11}H_{12}O_6$. Cyclopolic acid would then be a hexasubstituted benzene derivative, four of the substituents having C—C linkages and two of them having C—O linkages. Further, cyclopolide, which arises from cyclopolic acid by the loss of water, would involve the formation of the phthalide grouping,

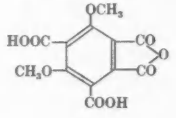
and the formation of a triacetate from cyclopolic acid would involve the closing of the phthalide ring, the acetylation of the OH group and the conversion of the CHO group into $CH(O.CO.CH_3)_2$, a similar mechanism explaining the formation of a diacetate from cyclopolide monomethyl ether.

The correctness of this hypothesis has been proved by a number of degradation experiments.

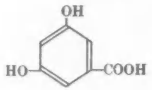
(*a*) Methyl cyclopolate monomethyl ether was oxidized with hot alkaline potassium permanganate. The main oxidation product, obtained in fairly good yield, was a tetracarboxylic acid, $C_{12}H_{10}O_{10}$ (IV), which was analysed as its anhydride and tetramethyl ester. The anhydride, $C_{12}H_8O_9$ (V),



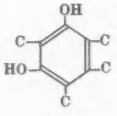
(IV)



(V)



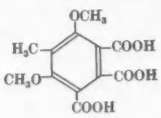
(VI)



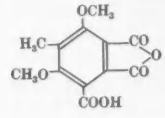
(VII)

colourless microprisms, m.p. 225·5–228° (decomp.), contained two methoxyl groups and titrated as a tetrabasic acid (di-acid mono-anhydride). The tetramethyl ester of (IV), $C_{16}H_{18}O_{10}$, a waxy solid, m.p. 44–46°, contained six methoxyl groups. The structure of the oxidation acid, $C_{12}H_{10}O_{10}$ (IV), 4:6-dimethoxybenzene-1:2:3:5-tetracarboxylic acid (cf. Birkinshaw, Raistrick & Ross, 1952), was conclusively proved as follows. It was heated in a stream of carbon dioxide-free nitrogen with hydriodic acid (*d*, 1·7) and red phosphorus. The acid was thus demethylated, three molecules of carbon dioxide were evolved and α-resorcylic acid (3:5-dihydroxybenzoic acid) (VI) was isolated in good yield and identified by comparison of it and of its dimethyl ether with authentic synthetic specimens. It therefore follows that cyclopolic acid must contain the skeleton structure (VII). The structure of the anhydride (V) follows because of the symmetrical nature of the free acid (IV).

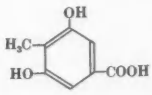
(*b*) Methyl cyclopolate monomethyl ether was oxidized with cold alkaline potassium permanganate. The main oxidation product was a tricarboxylic acid, $C_{12}H_{12}O_8$ (VIII), which was analysed as its anhydride, $C_{12}H_{10}O_7$ (IX). The anhydride, m.p. 198·5–199·5°, contained two methoxyl groups and titrated as a tribasic acid (mono-acid mono-anhydride). The structure of the oxidation acid,



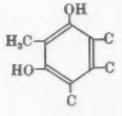
(VIII)



(IX)



(X)



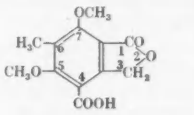
(XI)

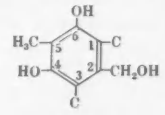
$C_{12}H_{12}O_8$ (VIII), 4:6-dimethoxy-5-methylbenzene-1:2:3-tricarboxylic acid, was conclusively proved as follows. On heating with hydriodic acid and red phosphorus the acid was demethylated, two molecules of carbon dioxide were evolved and 3:5-dihydroxy-*p*-toluic acid (X) was isolated and identified by comparison of it and of its methyl ester with authentic synthetic specimens. The skeleton structure of cyclopolic acid may therefore be expanded to (XI). The structure of the anhydride (IX) follows because of the symmetrical nature of the free acid (VIII).

(*c*) 3:5-Dihydroxy-*p*-toluic acid (X) was also isolated from the products of the fusion of cyclopolic acid with potassium hydroxide at 300°. It was identified by comparison of it, of its methyl ester and of the diacetate of its methyl ester with authentic synthetic specimens.

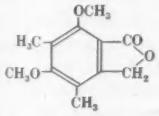
(*d*) Cyclopolide monomethyl ether was oxidized with cold alkaline potassium permanganate. 4-Carboxy-5:7-dimethoxy-6-methylphthalide (XII) was obtained in good yield as fine colourless needles, m.p. 198–199°, not depressed on admixture with an authentic synthetic specimen, m.p. 198–198·5° (for

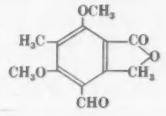
synthesis see p. 627). This oxidation product is also identical with the methyl ether of *iso*cyclopaldic acid (see p. 621). The skeleton structure of cyclopolic acid may therefore be further expanded to (XIII).

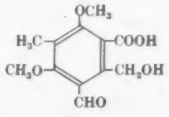

(XII)


(XIII)

(*e*) Cyclopolic acid was submitted to a Clemmensen reduction by boiling in aqueous hydrochloric acid solution with mercury-amalgamated zinc. The resulting reduction product, $C_{11}H_{12}O_4$, which no longer gave a precipitate with Brady's reagent or any colour with ferric chloride, was obtained as colourless lustrous needles, m.p. 202·5–203·5°. This substance, on methylation with dimethyl sulphate, acetone and anhydrous potassium carbonate, gave 5:7-dimethoxy-4:6-dimethylphthalide, $C_{12}H_{14}O_4$, (XIV), as colourless needles, m.p. 101·5–102°, not depressing the melting point of an authentic synthetic specimen (for synthesis see p. 627). It is clear that in this reduction the CHO group in cyclopolic acid is reduced to CH_3. Hence, since it has already been shown that the methyl group originally present in cyclopolic acid is in position 5 in (XIII), the CHO group in cyclopolic acid which gives rise to the second CH_3 group in (XIV) must be in position 3 in (XIII). Cyclopolide monomethyl ether must, therefore, have structure (XV), and the methyl ether of cyclopolic acid must have structure (XVI).

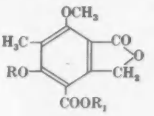

(XIV)

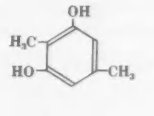

(XV)

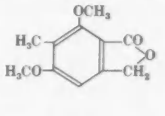

(XVI)

(*f*) The general orientation of cyclopolic acid was confirmed and its relation to cyclopaldic acid was indicated by a different line of approach. Cyclopolic acid was heated in air with potassium hydroxide and a little water at 150° until the melt ceased to give a precipitate with Brady's reagent. The resulting oxidation product, *iso*cyclopaldic acid, $C_{11}H_{10}O_6$ (XVII; $R=R_1=H$), was isolated as colourless needles, m.p. 223·5–224° (decomp.). The same acid was also obtained by heating cyclopaldic acid for a short time with aqueous 2N-sodium hydroxide. *iso*Cyclopaldic acid forms a monomethyl ether, colourless needles, m.p. 198–199°, which did not depress the melting point of authentic synthetic 4 - carboxy - 5 : 7 - dimethoxy - 6 - methylphthalide (XVII; $R=CH_3$, $R_1=H$), m.p. 198–198·5° (for synthesis see p. 627). This is the same monomethyl ether as is formed by the oxidation of cyclopolide monomethyl ether with cold alkaline potassium permanganate (see § *d*, p. 612, structure XII). *iso*Cyclopaldic acid also forms a monomethyl ether methyl ester, colourless needles, m.p. 139–139·5°, which did not depress the melting point of authentic synthetic 4 - carbomethoxy - 5 : 7 - dimethoxy - 6 - methylphthalide (XVII; $R=R_1=CH_3$), m.p. 139–139·5° (for synthesis see p. 627).

When *iso*cyclopaldic acid (XVII; $R=R_1=H$) was heated in nitrogen with hydriodic acid and red phosphorus it was demethylated, two molecules of carbon dioxide were evolved and the degradation product was identified as β-orcinol (XVIII) by comparison of it and of its diacetate with authentic synthetic specimens. On the other hand, when *iso*cyclopaldic acid was decarboxylated by heating with copper chromite and quinoline only one molecule of carbon dioxide was evolved and the methylated decarboxylated product was identified as 5:7-dimethoxy-6-methylphthalide (XIX), colourless needles, m.p. 171·5–172·5°, not depressed on admixture with an authentic synthetic specimen, m.p. 172–172·5° (for synthesis see p. 627). 5:7-Dimethoxy-6-methylphthalide (XIX) is of particular interest since it links cyclopolic acid and cyclopaldic acid with the lichen acid, barbatolic acid (XX). Suominen (1939), who isolated barbatolic acid from the lichen *Alectoria implexa* (Hoffm.) Nyl. f. *fuscidula* Arn., assigned structure (XX) to it. He esterified the carboxyl group in ring A with diazomethane and reduced the resulting methyl ester with zinc dust and hydriodic acid at 70°


(XVII)


(XVIII)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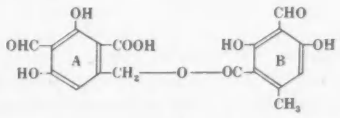
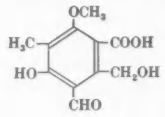

(XX)
Barbatolic acid

obtaining a reduction product, $C_{19}H_{20}O_8$, m.p. 189°, in which the aldehyde groups in rings A and B were reduced to methyl groups. The four hydroxyl groups in rings A and B were then methylated with diazomethane and the resulting methylated product was hydrolysed with ethanolic potassium hydroxide. He identified one of the resulting hydrolysis products as 5:7-dimethoxy-6-methylphthalide. Through the courtesy of Dr E. E. Suominen we received a specimen of the reduction product $C_{19}H_{20}O_8$, m.p. 189°, and have repeated his degradation process with some minor modifications. We isolated 5:7-dimethoxy-6-methylphthalide, m.p. 171·5°, not depressing the melting point of our synthetic 5:7-dimethoxy-6-methylphthalide, m.p. 172–172·5°, which has not previously been synthesized. Suominen (1939) gives the m.p. as 173·5°.

Having established the structural formula for the monomethyl ether of cyclopolic acid as (XVI) it now remains to decide which of the methoxyl groups—whether that in position 4 or that in position 6 in (XVI)—in this monomethyl ether is present as a



(XXI)
Cyclopolic acid

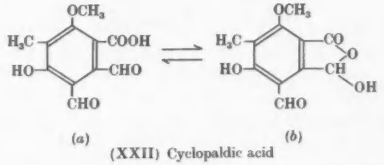
free hydroxyl group in cyclopolic acid itself. We believe that cyclopolic acid has structure (XXI), but defer consideration of the experimental evidence for this belief until the structural formula for cyclopaldic acid has been discussed.

CYCLOPALDIC ACID

Cyclopaldic acid, $C_{11}H_{10}O_6$, forms colourless fluffy needles, m.p. 224–225°, which sublime readily in a high vacuum at 170°. It contains one methoxyl group and one methyl group attached to carbon. It dissolves slowly in cold aqueous sodium bicarbonate and forms a yellow solution both in this alkali and in aqueous sodium hydroxide, in which it is quickly soluble. It titrates conductometrically as a dibasic acid. An aqueous solution of cyclopaldic acid gives with ferric chloride an intense ruby-red colour with no suggestion of blue; decolorizes bromine water without the formation of a precipitate; quickly gives a gelatinous orange precipitate with Brady's reagent; gives a negative reaction with Schiff's reagent. When solid cyclopaldic acid is exposed to ammonia vapour it rapidly turns brown, and if then dissolved in aqueous ammonia (*d*, 0·880) it gives an intense purple colour slowly fading to orange with a yellow fluorescence. Cyclopolic acid does not give this characteristic reaction.

The close structural relationship of cyclopaldic acid, $C_{11}H_{10}O_6$, to cyclopolic acid, $C_{11}H_{12}O_6$, is evident from the following experimentally established facts: (i) When cyclopolic acid is oxidized with potassium periodate in hot dilute sulphuric acid solution cyclopaldic acid is formed in fairly good yield. (ii) Cyclopaldic acid is smoothly converted into the isomeric *iso*cyclopaldic acid, $C_{11}H_{10}O_6$ (XVII; $R = R_1 = H$) when its solution in aqueous 2N-sodium hydroxide is boiled for a short time. The same compound is formed from cyclopolic acid by oxidation in air at 150° by heating with potassium hydroxide and a little water. (iii) Both methyl oxycyclopaldate methyl ether (XXVII) (see p. 615) and methyl cyclopolate monomethyl ether, on oxidation with hot alkaline potassium permanganate, yield the same oxidation product, 4:6-dimethoxybenzene-1:2:3:5-tetracarboxylic acid, (IV). (iv) Methyl oxycyclopaldate methyl ether, on mild oxidation with potassium permanganate and subsequent hydrolysis, yields 4:6-dimethoxy-5-methylbenzene-1:2:3-tricarboxylic acid (VIII), identical with the mild permanganate oxidation product from monomethyl cyclopolate methyl ether.

The simplest, and probably the only feasible, explanation for the formation of cyclopaldic acid from cyclopolic acid by the loss of two atoms of hydrogen, is that the carbinol group in cyclopolic acid (XXI) is converted into an aldehyde group in cyclopaldic acid. We therefore propose structure (XXII) for cyclopaldic acid, and, since some of its reactions are more readily explained by the tautomeric hydroxyphthalide form (*b*) than by the dialdehyde form (*a*), it becomes necessary to postulate that cyclopaldic acid may function in either form according to experimental conditions.

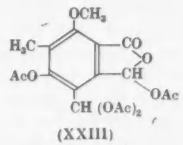


(*a*) (*b*)
(XXII) Cyclopaldic acid

The formation of the following derivatives and degradation products of cyclopaldic acid is readily explained in some cases by tautomeric form (*a*) and in other cases by tautomeric form (*b*).

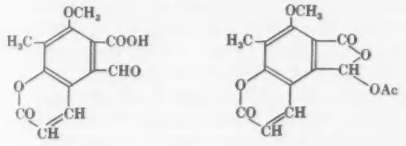
When cyclopaldic acid is heated at 150° for 0·5 hr. with anhydrous sodium acetate and acetic anhydride there is formed an acetylation product, $C_{19}H_{20}O_{11}$, in colourless needles, m.p. 159°. This substance, on acid hydrolysis, gives four molecules of acetic acid and is clearly the tetra-acetyl derivative of $C_{11}H_{10}O_6 + H_2O$. Unlike the parent substance, it no longer gives a ferric colour. Its structure (XXIII)

is readily derived from structure (XXIII*b*) for cyclopaldic acid.



(XXIII)

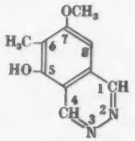
When a similar acetylation mixture was held at 180–190° for 6·5 hr. an entirely different product, $C_{15}H_{12}O_7$ (XXIV*b*), derived from (XXII*b*), was obtained in colourless needles, m.p. 197·5–198·5°. On acid hydrolysis, this substance yielded one molecule of acetic acid and a substituted coumarin, $C_{13}H_{10}O_6$, in colourless crystals, m.p. 275–277°, for which we propose structure (XXIV*a*), derived from (XXII*a*). It dissolves slowly in aqueous sodium bicarbonate to give a colourless solution, and at once in cold aqueous sodium carbonate or sodium hydroxide to give a yellow solution. It gives no ferric colour, but forms a yellow crystalline derivative with Brady's reagent in aqueous ethanolic solution.



(*a*) (*b*)

(XXIV)

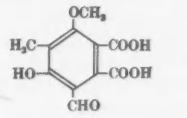
When a mixture of cyclopaldic acid, hydrazine and water is boiled for 15 min. there is formed, with the loss of one molecule of carbon dioxide, a condensation product, $C_{10}H_{10}O_2N_2$ (XXV), in pale-yellow prisms, m.p. 260–262° (decomp.). This



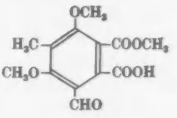
(XXV)

substance, which is a base forming a crystalline hydrochloride, gives a red-brown colour with ferric chloride in ethanol, but no precipitate with Brady's reagent. Its formation from cyclopaldic acid structure (XXII*a*) is readily explained, by assigning to it structure (XXV), as a derivative of phthalazine (4:5-benzopyridazine), i.e. 5-hydroxy-7-methoxy-6-methylphthalazine. The parent substance, phthalazine, has been synthesized by a similar reaction from phthaldialdehyde and hydrazine (Gabriel & Pinkus, 1893).

When cyclopaldic acid is oxidized at room temperature with alkaline hydrogen peroxide, oxycyclopaldic acid (XXVI) (6-formyl-5-hydroxy-3-methoxy-4-methylphthalic acid), colourless needles, m.p. 138° (decomp.), is formed in good yield. Unlike cyclopaldic acid, cyclopolic acid is not oxidized with alkaline hydrogen peroxide at room temperature and was recovered unchanged. Oxycyclopaldic acid gives a yellow solution in sodium hydroxide; a purplish red colour with ferric chloride; an immediate yellow precipitate with Brady's reagent; an anhydride (6-formyl-5-hydroxy-3-methoxy-4-methylphthalic anhydride), pale-yellow needles, m.p. 141°; and, on methylation with dimethyl sulphate and anhydrous potassium carbonate in acetone, it gives monomethyl oxycyclopaldate methyl ether (methyl-(2) 6-formyl-3:5-dimethoxy-4-methylphthalate) (XXVII).



(XXVI)



(XXVII)

This mono-ester is soluble in aqueous sodium bicarbonate, gives no colour with sodium hydroxide or ferric chloride, but gives a yellow crystalline precipitate with Brady's reagent. Oxidation of monomethyl oxycyclopaldate methyl ether (XXVII) with alkaline potassium permanganate under mild conditions gives 3-carbomethoxy-4:6-dimethoxy-5-methylphthalic acid, colourless plates, m.p. 135–137° (decomp.), which no longer gives a precipitate with Brady's reagent and on alkaline hydrolysis give the same 4:6-dimethoxy-5-methylbenzene-1:2:3-tricarboxylic acid (VIII) as was obtained by the mild permanganate oxidation of monomethyl cyclopolate methyl ether. Vigorous oxidation of monomethyl oxycyclopaldate methyl ether with hot alkaline potassium permanganate gives the same 4:6-dimethoxybenzene-1:2:3:5-tetracarboxylic acid (IV) as was obtained under the same conditions by the oxidation of monomethyl cyclopolate methyl ether.

The question as to which of the methoxyl groups in cyclopolic acid monomethyl ether (XVI)—whether that in position 6 or that in position 4—is present as a free hydroxyl group in cyclopolic acid itself, and hence also in cyclopaldic acid, resolves itself into a decision whether cyclopolic acid is best regarded as a substituted *ortho*-hydroxycarboxylic acid or as an *ortho*-hydroxyaldehyde. The experimental evidence at present available favours the latter conclusion as shown in structure (XXI) for cyclopolic acid and (XXII*a* and *b*) for cyclopaldic acid, and can be summarized as follows.

(1) Cyclopolic acid gives with aqueous ferric chloride an intense stable purple colour which is almost indistinguishable from that given by both salicylic acid and salicylaldehyde with the same reagent. However, two derivatives of cyclopolic acid have been described in which the CHO group has been modified, namely its Clemmensen reduction product, 5-hydroxy-7-methoxy-4:6-dimethylphthalide ($CHO \rightarrow CH_3$) (see p. 613), and its thiosemicarbazone ($CHO \rightarrow CH{=}N.NH.CS.NH_2$). The former derivative gives no ferric colour and the latter only an orange colour. This evidence clearly supports the view that cyclopolic acid is an *ortho*-hydroxyaldehyde, since, if it were an *ortho*-hydroxycarboxylic acid its ferric reaction should not be profoundly affected by modification of the CHO group in another part of the molecule.

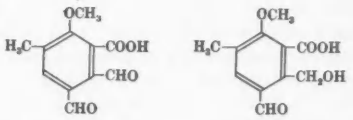
(2) The formation of a substituted coumarin (XXIV*b*) from cyclopaldic acid by heating with acetic anhydride and sodium acetate is an example of the well known Perkin synthesis of coumarins, the simplest example of which is the formation, by the same process, of coumarin itself from salicylaldehyde (*ortho*-hydroxybenzaldehyde).

(3) Most *ortho*-hydroxyaldehydes dissolve in cold sodium hydroxide with the formation of yellow solutions. Cyclopaldic acid and cyclopolide give this reaction although, curiously enough, cyclopolic acid forms a colourless solution.

(4) 2:6-Dichloroquinone chloroimide quickly reacts with a solution of cyclopolic acid in buffer (pH 9·2) to give a pure blue colour, while the yellow solution of cyclopaldic acid in the same buffer turns more slowly blue-green with the same reagent. According to Gibbs (1927) and Davidson, Keane & Nolan (1943) this test is specific for phenols with the para position free or occupied by the carboxyl group. The reaction with 2:6-dichloroquinone chloroimide has been used previously in this laboratory in connexion with similar structural problems in the cases of geodin and erdin (Calam, Clutterbuck, Oxford & Raistrick, 1947) and of mycophenolic acid (Birkinshaw, Bracken, Morgan & Raistrick, 1948).

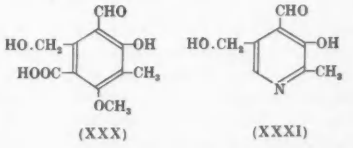
In addition to cyclopolic acid and cyclopaldic acid two other mould metabolic products are known in which all six positions in the benzene ring are substituted. Citrinin from *P. citrinum* Thom (Hetherington & Raistrick, 1931; Coyne, Raistrick & Robinson, 1931), from *Aspergillus terreus* Thom (Raistrick & Smith, 1935), and a number of other moulds, has been synthesized by Cartwright, Robertson & Whalley (1949). Mycophenolic acid, first isolated by Alsberg & Black in 1913 from culture filtrates of *P. stoloniferum* Thom, has more recently been reported from a large number of other species and strains in the *P. brevi-compactum* Dierckx series (Clutterbuck, Oxford, Raistrick & Smith, 1932; Clutterbuck & Raistrick, 1933; Birkinshaw *et al.* 1948; Birkinshaw *et al.* 1952). The close interrelationships in structure between cyclopolic acid, cyclopaldic acid, citrinin and mycophenolic acid have been discussed in detail by Raistrick (1950).

Cyclopaldic acid has a very pronounced fungistatic potency. When tested against the conidia of *Botrytis allii* Munn. in Czapek-Dox medium at pH 3·5 by the technique described by Brian & Hemming (1945) it produced 95–100% inhibition of germination at a concentration of 2·5μg./ml. We found that gladiolic acid, a metabolic product of *P. gladioli* Machacek, described by Brian, Curtis & Hemming (1948), tested at the same time and under the same conditions, gave the same inhibition at a concentration of 10·0μg./ml., a figure which is in good agreement with that previously recorded for gladiolic acid by its describers, i.e. 7·8μg./ml. On the other hand, cyclopolic acid had no effect on the germination of *B. allii* spores at a concentration of 160μg./ml. and gave only a 10% inhibition at a concentration of 320μg./ml., while dihydrogladiolic acid gave no inhibition at 320μg./ml. These findings are of particular interest in view of the close structural relationship between gladiolic acid, structure (XXVIII) established by Raistrick & Ross (1952) and independently by Grove (1952), and cyclopaldic acid, which is clearly 4-hydroxygladiolic acid, and also between dihydrogladiolic acid, structure (XXIX) (Raistrick & Ross, 1952), and cyclopolic acid, which is 4-hydroxydihydrogladiolic acid.



(XXVIII) Gladiolic acid (XXIX) Dihydrogladiolic acid

There is also a striking structural resemblance, which may be of some biological significance, between cyclopolic acid (XXX) and members of the vitamin B_6 group, in particular pyridoxal (XXXI).



(XXX) (XXXI)

EXPERIMENTAL

All melting points are uncorrected. Determinations of methoxyl and equivalent by titration were carried out in this department. All other micro-analyses were carried out by Weiler and Strauss, Oxford.

Table 1. *Production of cyclopolic and cyclopaldic acids by strain no.* 85

Incubation period days	Glucose (by polarimeter) (%)	pH	Total ethyl acetate extract (g./flask)	Cyclopolic acid (mg./flask)	Cyclopaldic acid (mg./flask)
6	2·57	3·0	0·18	22	5
9	1·15	3·0	0·30	100	15
13	0·35	3·3	0·36	145	46
16	0·16	3·7	0·31	142	30
20	—	5·4	0·27	110	—
22	—	5·9	0·27	44	24

History of cultures

Two mould cultures were used during this work. Their history is as follows.

P. cyclopium Westling var. *album* G. Smith, L.S.H.T.M. cat. no. 85, was isolated from a mouldy banana skin in May 1947. It was identified and a full description of it was given by our colleague, Mr G. Smith (see Smith, 1951). It is morphologically a typical strain of *P. cyclopium* in all respects except that it is almost pure white in colour and there is an almost total lack of green conidial pigment.

P. cyclopium Westling, L.S.H.T.M. cat. no. 77, was isolated from a mouldy cooked bone in April 1947. It was identified by Mr G. Smith as a typical green-sporing strain of *P. cyclopium* Westling.

Both cultures were isolated originally by Mrs S. Marcus of this department.

Cultural conditions

Quantities of 350 ml. of Raulin-Thom solution (glucose, 75·0 g.; tartaric acid, 4·0 g.; ammonium tartrate, 4·0 g.; $(NH_4)_2HPO_4$, 0·6 g.; $(NH_4)_2SO_4$, 0·25 g.; K_2CO_3, 0·6 g.; $MgCO_3$, 0·4 g.; $FeSO_4.7H_2O$, 0·07 g.; $ZnSO_4.7H_2O$, 0·07 g.; distilled water, 1500 ml.) were distributed in a number of 1 l. conical flasks, plugged with cotton wool, and sterilized. Batches of fifty flasks were inoculated with a spore suspension in sterile distilled water of either strain no. 85 or no. 77 of *P. cyclopium* which had been cultivated on malt-agar slopes at 24° for 7 days, four flasks being inoculated from each malt-agar slope. The inoculated flasks were incubated at 24° in the dark.

Strain no. 85. Growth of this strain was good and after 2 days had extended over the whole surface of the medium. After 5 days the mycelium became convoluted, the upper surface being white to cream coloured, the lower surface yellow to light brown, and wherever it was exposed to the air it slowly turned brown and finally black after 21 days. Otherwise there was little change in the general appearance of the mycelium from 5 days onwards. The culture fluid was pale yellow after 7 days, brownish yellow after 14 days and dark brown after 21 days. It gave a strong brownish-red colour with aqueous $FeCl_3$ from 13 days' incubation onwards.

Strain no. 77. Growth of this strain was good. After 7 days the mycelium was very convoluted, the upper surface being mottled green, the reverse being buff coloured. After 14 and 21 days the only marked change in appearance was that the upper surface was much darker green in colour and the reverse was also darker in colour with some brick coloured areas. The culture fluid was pale yellow after 7 days, yellow after 14 days and brown after 21 days. It gave a strong clear reddish-brown to ruby colour with aqueous $FeCl_3$ after 14 days' incubation.

Neither culture fluid inhibited at a dilution of 1:10 the growth of the Oxford H strain of *Staphylococcus aureus* or the National Collection of Type Cultures strain no. 86 of *Bacterium coli* (*Escherichia coli*) at any period of growth up to 14 days.

Course of metabolism of strain no. 85

A number of flasks from a batch inoculated with strain no. 85 were harvested at frequent intervals. The course of the metabolism was followed by measuring the residual glucose polarimetrically and the approximate yields of cyclopolic and cyclopaldic acids by the isolation methods described below. The results obtained are summarized in Table 1.

It will be seen that the yields of cyclopolic and cyclopaldic acids reach a maximum after 13–16 days' incubation and thereafter decrease quickly. For this reason, in the bulk preparation of cyclopolic and cyclopaldic acids, all flasks of strain no. 85 were harvested after 14 days' incubation at 24°.

Isolation of cyclopolic and cyclopaldic acids

The culture fluid from each batch of fifty 1 l. flasks of either strain no. 85 or strain no. 77 was separated from the mycelium by straining through muslin and was then clarified by filtration after the addition of about 1 g./l. of kieselguhr.

(i) *Strain no.* 85. The clear filtrate (15 l.) after 14 days' growth at 24° was divided into two portions and extracted three times, without acidification, in two 10 l. bottles with ethyl acetate (2 l. each time). After the third extraction the culture filtrate gave only a slight ferric reaction. The combined filtered ethyl acetate extracts (12 l.) were evaporated at about 110 mm. pressure at 40–50° to a residual volume of 200–300 ml. when a quantity of white to cream solid had already separated. The mixture was held at room temperature for some days when the solid material was separated by filtration, washed with a little cold ethyl acetate, and dried. The dried solid consisted of fairly pure cyclopolic acid, the melting point varying in different batches from 139 to 147° (decomp.) (see Table 2, column 4, 1st crop).

Further evaporation of the ethyl acetate mother liquors, usually from two or more batches combined, gave a second crop of less pure cyclopolic acid (see Table 2, column 5, 2nd crop).

The final ethyl acetate mother liquors were evaporated to dryness under reduced pressure giving a partly solid, partly gummy residue which was triturated with cold ether (100–150 ml.) until all the gum had dissolved. The solid was separated by filtration, washed with ether and dried. It consisted essentially of cyclopaldic acid, the melting point of which varied between 183 and 212° (see Table 2, column 6).

Table 2. *Yields of crude cyclopolic and cyclopaldic acids isolated from batches of fifty flasks of strain no.* 85 *grown for* 14 *days at* 24°

Batch no.	Glucose (by polarimeter) (%)	pH	Cyclopolic acid 1st crop (g.)	Cyclopolic acid 1st crop M.p. (decomp.) (°)	Cyclopolic acid 2nd crop (g.)	Cyclopolic acid 2nd crop M.p. (decomp.) (°)	Cyclopaldic acid (g.)	Cyclopaldic acid M.p. (°)
1	0·21	3·3	5·8	143–147	—	—	4·5	183–195
2	0·17	3·5	5·5	141–142				
3	0·32	3·4						
4	0·09	3·5	10·2	147	9·5	130–137	4·0	209–212
5	0·12	3·7						
6	0·13	3·7	3·6	139–140				
7	0·08	3·8	—	—	—	—	3·4	193–212

Table 3. *Production of ether extractable metabolic products by strain no.* 77

Batch no.	Glucose (by polarimeter) (%)	pH	Crop I (g.)	Crop I M.p. (°)	Crop II (g.)	Crop II M.p. (°)	Crop III (g.)	Crop III M.p. (°)	Uncrystallizable ether residue (g.)
1	0·38	3·6	2·49	148–151 (decomp.)	0·61	150–240	—	—	3·3
2	0·36	3·6	4·06	142–145 (decomp.)	1·40	140–175	—	—	3·19
3	0·17	3·6	4·40	142–144 (decomp.)	0·80	172–174	0·08	180–200	3·45
4	0·17	3·6	4·20	138–142 (decomp.)	0·75	132–140	—	—	3·5

Evaporation of the ether mother liquors gave a considerable quantity (41 g. from 350 flasks) of a brown semi-solid, partly crystalline gum from which no other pure crystalline substance could be isolated.

The approximate yields of cyclopolic and cyclopaldic acids obtained from batches of fifty flasks of strain no. 85, incubated for 14 days at 24°, are shown in Table 2.

It will be seen from Table 2 that from a total of 350 flasks 34·6 g. of cyclopolic acid (equal to 4·9 g./fifty flasks) and 11·9 g. of cyclopaldic acid (equal to 1·7 g./fifty flasks) were isolated.

The metabolism solution, which, in batches 1–4 was light yellow in colour, became darker in batches 5–6, and in batch 7 was deep brown in colour. This deepening in colour follows a steady rise in pH from batch 1 to batch 7, and is clearly connected with the decreased yield of cyclopolic acid in batch 6 and its absence from batch 7.

(ii) *Strain no.* 77. The clear culture filtrate (15 l.), after 14 days' growth at 24°, was acidified with 5N-HCl (150 ml.). The acidified filtrate was divided into two equal portions and each portion was extracted three times with ether (2 l. + 2 l. + 1·5 l.), centrifuging being necessary to obtain good separation. The extracted filtrate now gave only a brownish ferric colour. The combined ether extracts were evaporated. Crystalline material began to separate after evaporating to about 400 ml. and evaporation was continued to about 150 ml. The concentrate and crystals were held at 0° for 3 days and filtered, giving crop I. Crops II and III were obtained by further concentration of the mother liquor. On drying the final ether mother liquor there remained a dark-red oily residue from which no further crystalline material could be obtained. The yields of extracted metabolic products from batches of fifty flasks are given in Table 3.

The crude extracted metabolic products (crops I–III) consisted of a mixture of cyclopolic acid and cyclopaldic acid which proved much more difficult to separate in a state of purity than the ethyl acetate extracts from strain no. 85. The simplest method of fractionation was as follows. Crop I from batch 4 (4·20 g.; m.p. 138–142°) was well stirred with water (100 ml.) at 50° and the undissolved portion was separated by filtration. It was then dissolved in boiling water and filtered from a trace of dark-brown material. On cooling the filtrate, brownish crystals (0·37 g.) separated, m.p. 210–213°, raised to 224° on further purification and not depressed on admixture with cyclopaldic acid from strain no. 85.

Two crops of colourless crystals (0·91 g., m.p. 140–144° (decomp.); 0·74 g., m.p. 142–147° (decomp.)) were obtained on cooling the aqueous filtrate obtained at 50°. Similarly, three crops of crystals were obtained on evaporation of the aqueous mother liquors from the crude cyclopaldic acid, giving in all a total of 2·84 g. of crude cyclopolic acid.

Direct comparison of strains no. 85 *and* 77

(By Mr M. M. Abou Zeid)

A direct comparison of the yields of cyclopolic and cyclopaldic acids produced by strains nos. 85 and 77 was made some 2 years after the completion of the work already described. The same medium and cultural conditions were used but the metabolic products were isolated in each case by the ethyl acetate extraction method described on p. 617. A summary of the results obtained follows.

Strain no. 85. Fifty flasks; incubation period, 14 days; pH, 3·1; residual glucose by polarimeter, 0·44%; weight of dry mycelium, 188 g.; crude cyclopolic acid obtained, 3·12 g., m.p. 147–148° (decomp.); crude cyclopaldic acid obtained, 1·84 g., m.p. 207–209°, which on crystallization from boiling water (300 ml./g. + charcoal) gave cyclopaldic acid as colourless needles, 0·96 g., m.p. 223–224°; uncrystallizable residue from ether mother liquors, 6·55 g.

Strain no. 77. Fifty flasks; incubation period, 14 days; pH, 3·3; residual glucose by polarimeter, 0·46%; weight of dry mycelium, 209 g.; crude cyclopolic acid obtained, 12·4 g., m.p. 147–148° (decomp.); no cyclopaldic acid could be isolated; uncrystallizable residue from ether mother liquors, 9·02 g.

Cyclopolic acid

Purification. Crude cyclopolic acid (11·3 g.) was recrystallized by solution in boiling ethyl acetate (0·5 l.) and the addition of benzene (1·5 l.). Yield of pure cyclopolic acid 7·0 g.

General properties. Cyclopolic acid crystallizes in colourless plates, m.p. 147–148° (decomp.). When finely ground it melts at 138–140° (decomp.). The acid melts with effervescence and loss of water to a clear straw-coloured liquid which does not reset on cooling. (Found: C, 55·0; H, 5·1; OCH_3, 12·9; *C*-CH_3, 4·5 = 0·71 *C*-CH_3 groups; equivalents, by conductometric titration, 236, 120. $C_{11}H_{12}O_6$ requires C, 55·0; H, 5·0; $1OCH_3$, 12·9; $1C$-CH_3, 6·3%; equivalent, titrating as a monobasic acid, 240; as a dibasic acid, 120.) The acid (*c*, 2·5 in methanol) showed no optical activity in a 2 dm. tube. It is soluble in water, methanol, ethyl acetate, ether and acetone, but is almost insoluble in benzene and light petroleum. It dissolves at once with effervescence in cold aqueous $NaHCO_3$ to a colourless solution. Its solution in cold 2N-NaOH is also colourless. Its aqueous solution gives an intense stable purple colour, almost indistinguishable from that given by salicylic acid, with aqueous $FeCl_3$. Its ethanolic solution gives a similar colour, but somewhat bluer in shade, with ethanolic $FeCl_3$. Brady's reagent (0·32% 2:4-dinitrophenylhydrazine in aqueous 2N-HCl) quickly gives a deep-yellow crystalline precipitate. It reduces ammoniacal $AgNO_3$ on warming, but it does not reduce Fehling's solution on heating for several minutes. It gives a negative reaction with Schiff's reagent. Under the conditions described for the test on cyclopaldic acid (see p. 624) no colour is obtained with conc. NH_3 (*d*, 0·880). With bleaching powder solution it gives a yellow colour fading to colourless in a few minutes. It gives with bromine water a turbidity soluble in excess of the reagent. When dissolved in buffer solution, pH 9·2, and treated with a few drops of an aqueous suspension of 2:6-dichloroquinone chloroimide a deep-blue colour is formed in 2 min. which is stable for 3 days.

Functional derivatives

Cyclopolic acid mono-2:4-dinitrophenylhydrazone. Brady's reagent (40 ml.) was added to a cold solution of cyclopolic acid (100 mg.) in water (10 ml.). The resulting deep-yellow precipitate of microprisms was separated by filtration, washed and dried (weight 113 mg.), m.p. 190·5–191° (decomp.) not changed on recrystallization from ethanol. (Found: C, 48·5; H, 3·6; N, 13·1. $C_{17}H_{16}O_9N_4$ requires C, 48·6; H, 3·8; N, 13·3%.) *Cyclopolic acid mono-2:4-dinitrophenylhydrazone* gave a red colour with ethanolic NaOH (Neuberg reaction) confirming a mono-derivative.

Cyclopolic acid thiosemicarbazone. A solution of cyclopolic acid (120 mg.) in methanol (1·5 ml.) was added to a hot solution of thiosemicarbazide (45 mg.) and anhydrous sodium acetate (60 mg.) in 0·25N-HCl (2 ml.), and the whole was heated at 100° for 5 min. It was then cooled, diluted with water (40 ml.) and held at room temperature for 3 days. The hard slightly brown solid which separated was collected (weight 89 mg.), m.p. 180·5–182° (decomp.). Recrystallization from ethanol (+charcoal) yielded colourless microprisms of the same melting point. (Found: C, 45·9; H, 5·0; N, 13·6; S, 10·3. $C_{12}H_{15}O_5N_3S$ requires C, 46·0; H, 4·8; N, 13·4; S, 10·2%.) *Cyclopolic acid thiosemicarbazone* is readily soluble in cold aqueous $NaHCO_3$. Its ethanolic solution gives an orange colour with ethanolic $FeCl_3$.

Acetylation of cyclopolic acid. Formation of cyclopolide triacetate. A mixture of cyclopolic acid (0·50 g.), sodium acetate (1·0 g.) and acetic anhydride (2 ml.) was refluxed for 0·5 hr. On adding water a light-brown solid (0·71 g.), m.p. 110–118° separated. Two crystallizations from 60% aqueous methanol gave colourless needles (0·32 g.), m.p. 117°. (Found: C, 55·8; H, 4·8; OCH_3, 8·4; $COCH_3$, 33·8. $C_{17}H_{18}O_9$ requires C, 55·7; H, 5·0; $1OCH_3$, 8·5; $3COCH_3$, 35·2%.) *Cyclopolide triacetate* is insoluble in cold 2N-NaOH except on long standing. Its ethanolic solution gives no colour with ethanolic $FeCl_3$ nor any precipitate with Brady's reagent. When this acetylation was carried out at 180–190° for 6·5 hr. the product was still cyclopolide triacetate. Under these conditions cyclopaldic acid gave a substituted coumarin (see p. 624).

A solution of cyclopolic acid (0·50 g.) in pyridine (5 ml.) and acetic anhydride (5 ml.) was held at 37° for 4 days. The solid formed (0·71 g.), m.p. 112–113°, on the addition of water, was collected and crystallized from aqueous methanol yielding thick colourless needles (0·41 g.), m.p. 116·5–117°, not depressed on admixture with cyclopolide triacetate prepared with sodium acetate and acetic anhydride.

Cyclopolide. (*a*) From cyclopolide triacetate. Cyclopolide triacetate (0·40 g.) was refluxed with aqueous 2N-H_2SO_4 (20 ml.) for 4 hr. The resulting light-brown solid (0·35 g.), m.p. 163–165°, was collected and crystallized twice from aqueous 66% ethanol giving colourless delicate needles, m.p. 169°, which reset and remelted at the same temperature. Sublimation in a high vacuum at 120–140° gave fluffy needles of the same melting point. (Found: C, 59·6, 59·5; H, 4·6, 4·6; OCH_3, 14·0; *C*-CH_3, 6·8; mol.wt. (Rast) 232. $C_{11}H_{10}O_6$ requires C, 59·4; H, 4·5; $1OCH_3$, 14·0; $1C$-CH_3, 6·8%; mol.wt. 222.) The trivial name *cyclopolide* is given to this substance despite the fact that it is not strictly accurate nomenclature. Cyclopolide is almost insoluble in cold water, but dissolves readily in cold aqueous $NaHCO_3$. It dissolves in cold 2N-NaOH to a pale-yellow solution which deepens in colour on warming. Its ethanolic solution gives a ruby-red colour with ethanolic $FeCl_3$, very similar to that given by cyclopaldic acid in ethanol, and an immediate orange precipitate with Brady's reagent. When cyclopolide is warmed with ammoniacal $AgNO_3$ a black precipitate is slowly formed but without the formation of a distinct silver mirror.

Cyclopolide was also obtained, though in much smaller yields, by each of the following methods.

(*b*) Cyclopolide triacetate (2·8 g.) was refluxed for 2 hr. with 2N-HCl (20 ml.) and methanol (40 ml.). Fractionation, from ether and aqueous methanol, of the brown gum remaining after removal of the solvent *in vacuo* at 50° yielded cyclopolide (0·37 g.), m.p. 161–164°.

(*c*) A solution of cyclopolic acid (0·20 g.) in 2N-H_2SO_4 (17 ml.) was refluxed for 3·5 hr. A light-brown solid (0·15 g.) separated. On fractional crystallization from aqueous ethanol (+charcoal) it yielded needles (30 mg.), m.p. 152°, which were purified by fractional sublimation in a high vacuum giving cyclopolide (11 mg.), m.p. 164–165°.

(*d*) Cyclopolic acid (0·20 g.) was heated at 155° for 20 min. and then at 170° for 2 min. Fractional sublimation of the melt in a high vacuum gave at 120–140° 4 mg. of cyclopolide, m.p. 165°. The melting point of the specimens of cyclopolide from methods (*b*), (*c*) or (*d*) was not depressed on admixture with pure cyclopolide, m.p. 169° from method (*a*).

Derivatives of cyclopolide. (*a*) *Cyclopolide monomethyl ether.*[1] An excess of ethereal diazomethane was added to a

solution of cyclopolide (94 mg.) in acetone (5 ml.). After standing overnight the solution was filtered and evaporated to dryness. The residual slightly gummy solid (102 mg.) was crystallized twice from aqueous methanol giving *cyclopolide monomethyl ether* as long colourless needles (70 mg.), m.p. 169–170°, resetting and remelting at the same temperature. (Found: C, 61·1, 61·0; H, 5·1, 5·1; OCH_3, 26·1. $C_{12}H_{12}O_5$ requires C, 61·0; H, 5·1; $2OCH_3$, 26·3%.) Cyclopolide monomethyl ether is insoluble in aqueous $NaHCO_3$ and does not dissolve in cold 2N-NaOH except on long standing. It gives no colour with $FeCl_3$ in ethanolic solution.

(*b*) *Mono-2:4-dinitrophenylhydrazone of cyclopolide monomethyl ether.* Brady's reagent (40 ml.) was added to a hot solution of cyclopolide monomethyl ether (48 mg.) in ethanol (8 ml.). The resulting orange gelatinous precipitate (76 mg.) was collected and crystallized from pyridine giving *mono-2:4-dinitrophenylhydrazone of cyclopolide monomethyl ether* as silky deep-orange needles (65 mg.) which on heating decompose progressively from 280 to 360° without melting. (Found: C, 52·2; H, 3·8; N, 13·5. $C_{18}H_{16}O_8N_4$ requires C, 51·9; H, 3·9; N, 13·5%.)

(*c*) *Diacetate of cyclopolide monomethyl ether.* The crude product (110 mg.) resulting from the alkaline (2N-KOH) hydrolysis of the methyl ether of methyl cyclopolate (320 mg.) was refluxed for 0·5 hr. with anhydrous sodium acetate (70 mg.) and acetic anhydride (0·3 ml.). The light-brown solid (120 mg.), formed on the addition of water, was collected, crystallized twice from light petroleum, b.p. 60–80°, giving crystals (26 mg.), m.p. 128–129°, and then twice from aqueous methanol giving the *diacetate of cyclopolide monomethyl ether* as colourless blades (12 mg.), m.p. 133·5–134°. (Found: C, 57·2; H, 5·5; OCH_3, 18·6. $C_{16}H_{18}O_8$ requires C, 56·8; H, 5·4; $2OCH_3$, 18·3%.) Its ethanolic solution gives no precipitate with Brady's reagent. The diacetate of cyclopolide monomethyl ether (5 mg.) was refluxed for 2 hr. with 2N-H_2SO_4 (1 ml.). On cooling, light-brown needles (2·5 mg.) separated, m.p. 159° not depressed on admixture with authentic cyclopolide monomethyl ether.

Methylation of cyclopolic acid with diazomethane

Formation of methyl cyclopolate monomethyl ether. An excess of ethereal diazomethane was added to a solution of cyclopolic acid (0·50 g.) in acetone (10 ml.) giving an immediate strong effervescence. After standing overnight the solution was filtered and evaporated to dryness giving a pale-amber gum (0·55 g.) which partially solidified on standing. The crystalline portion was separated from insoluble gum by extraction with light petroleum, b.p. 40–60°, which, on evaporation, gave small plates (0·22 g.), m.p. 80°. This material was crystallized from water giving *methyl cyclopolate monomethyl ether* (0·13 g.) as colourless plates, m.p. 87·5–88°. (Found: C, 58·3, 58·4; H, 6·0, 5·9; OCH_3, 34·4; active H atoms (in anisole), 0·63 atom. $C_{13}H_{16}O_6$ requires C, 58·2; H, 6·0; $3OCH_3$, 34·7%.) An aqueous solution of the ester gives no colour with $FeCl_3$, but quickly gives a yellow precipitate with Brady's reagent.

In the hope of obtaining cyclopolic acid monomethyl ether, the total crude ester (0·59 g.), resulting from a second methylation of cyclopolic acid (0·50 g.) with ethereal diazomethane, was dissolved in methanol (5 ml.) and treated with 5 ml. of methanolic potash (2 g. KOH in methanol 10 ml.). After holding for 4 days at room temperature water (10 ml.) and 2N-HCl (8 ml.) were added. Methanol was removed *in vacuo* and the aqueous solution was extracted with ether. On removal of the solvent the residue slowly crystallized (0·52 g.). This product could not be satisfactorily purified. It was characterized, however, by conversion into a mono-2:4-dinitrophenylhydrazone which was obtained in good yield.

Mono-2:4-dinitrophenylhydrazone of cyclopolic acid monomethyl ether. Brady's reagent (10 ml.) was added to a solution in water (10 ml.) of the above crude cyclopolic acid monomethyl ether (20 mg.). The yellow crystalline precipitate (25 mg.), m.p. 287° (decomp.), which readily formed, was collected and crystallized from methanol giving the *mono-2:4-dinitrophenylhydrazone of cyclopolic acid monomethyl ether* as yellow needles, m.p. 287° (decomp.). (Found (*a*) on sample dried at room temperature in a high vacuum: C, 47·9; H, 4·5; N, 12·8. $C_{18}H_{16}O_8N_4.1H_2O$ requires C, 47·8; H, 4·5; N, 12·4%; (*b*) on sample dried at 100° *in vacuo*, loss in weight 3·7; $C_{18}H_{16}O_8N_4.1H_2O$ requires H_2O, 4·0%; C, 49·9; H, 4·4; N, 12·9. $C_{18}H_{16}O_8N_4$ requires C, 49·8; H, 4·2; N, 12·9%.) Both the hydrated and anhydrous forms are soluble in cold saturated aqueous $NaHCO_3$ and give a red Neuberg reaction confirmatory of a mono-2:4-dinitrophenylhydrazone.

Conversion of cyclopolic acid into cyclopaldic acid

Cyclopolic acid, m.p. 147° (decomp.), (0·10 g.), potassium periodate (0·10 g.) and N-H_2SO_4 (10 ml.) were boiled under reflux for 5 min. giving a pale-brown solution which then clouded with some decomposition. On cooling, needles (36 mg.), m.p. 215–219°, separated. Sublimation in a high vacuum at 150–160° gave colourless crystals of cyclopaldic acid (26 mg.), m.p. 222–223° with sublimation, unchanged on mixing with cyclopaldic acid, m.p. 224–225°, isolated directly from the culture filtrate of *P. cyclopium* var. *album* no. 85. The mixture reset on cooling and remelted at the same temperature.

Degradation products of cyclopolic acid

A. *Clemmensen reduction of cyclopolic acid and methylation of reduction product. Isolation of 5:7-dimethoxy-4:6-dimethylphthalide (XIV)*

Granulated zinc (20 g.) was immersed in an aqueous solution of $HgCl_2$ (40 ml. of a 5% solution) for 1 hr. Part of the washed amalgam (12 g.) was suspended in conc. HCl (15 ml.) and heated under reflux. Cyclopolic acid (0·50 g.), dissolved in hot water (15 ml.), was gradually added during 0·75 hr., and during the reduction two additions of 5 ml. of conc. HCl were made at hourly intervals. The course of the reduction was followed by periodic addition of Brady's reagent to a few drops of the reaction liquid which gave at first an orange, and later a yellow precipitate. After 3·5 hr. no precipitate was obtained with Brady's reagent and the reduction was stopped. The reaction mixture was held overnight, when the gummy reduction product had solidified and a further quantity of colourless solid had separated. The total product was mechanically separated from the residual Zn, filtered, washed with water and dried, wt. 0·36 g., m.p. 193–199°. Since no satisfactory solvent could be found for the crystallization of the crude reduction product the whole of it was fractionally sublimed in a high vacuum, and at 160–170° gave a colourless solid (60 mg.), m.p. 202–203°, which, on crystallization from benzene, gave *5-hydroxy-7-methoxy-4:6-dimethylphthalide* as colourless lustrous needles,

m.p. 202·5–203·5°, resetting and remelting at the same temperature. (Found: C, 63·7; H, 6·1; OCH_3, 14·9. $C_{11}H_{12}O_6$ requires C, 63·5; H, 5·8; $1OCH_3$, 14·9%.) It is soluble in cold aqueous 2N-Na_2CO_3 and its methanolic solution gives no colour with $FeCl_3$. The reduction product (25 mg.) was methylated in the usual way with dimethyl sulphate, acetone and anhydrous K_2CO_3. The crude methylation product (22 mg.), m.p. 98–99°, was crystallized from light petroleum, b.p. 60–80°, giving colourless needles (15 mg.), of 5:7-*dimethoxy*-4:6-*dimethylphthalide*, m.p. 101·5–102°, resetting and remelting at the same temperature and not depressed on admixture with an authentic synthetic specimen, m.p. 98–100° (see p. 627). (Found: C, 64·7; H, 6·3; OCH_3, 28·1. $C_{12}H_{14}O_4$ requires C, 64·9; H, 6·4; $2OCH_3$, 27·9%.) It does not dissolve in cold 2N-NaOH.

B. *Potash fusion of cyclopolic acid at 300°. Formation of 3:5-dihydroxy-4-methylbenzoic acid*

KOH (2·5 g.) and water (0·5 ml.) were heated to 150° in a nickel crucible floating on a Wood's metal bath. Cyclopolic acid (0·50 g.) was added, and during 15 min. the bath temperature was raised to 300° and maintained at this temperature for a further 10 min. when the original vigorous reaction had practically ceased. The dark-brown fluid melt was cooled, water (50 ml.) was added and the resultant solution was adjusted to pH 2 with 2N-HCl. An aqueous solution of $CaCl_2$ (0·2 g.) was added. After standing overnight the small precipitate of calcium oxalate was filtered off and the filtrate was extracted with ether. On removal of the solvent there remained a pale-brown resinous solid (0·33 g.) which was purified by fractional sublimation in a high vacuum, giving at 150–180° a small gummy upper layer which was not identified and a light-brown solid lower layer (0·18 g.), m.p. 254–259° (decomp.). This lower layer was purified by repeated crystallization from water giving colourless needles of 3:5-dihydroxy-4-methylbenzoic acid (3:5-dihydroxy-*p*-toluic acid), m.p. 269–270° (decomp.) not depressed on admixture with an authentic synthetic specimen (see p. 626). (Found (*a*) on sample dried to constant weight at room temperature: C, 53·9; H, 5·4; H_2O, 5·2, 5·4; OCH_3, nil. Calc. for $C_8H_8O_4 \cdot \frac{1}{2}H_2O$: C, 54·2; H, 5·1; H_2O, 5·1%. (*b*) On sample dried to constant weight at 100°: C, 57·4, 57·2; H, 4·7, 4·8; equivalent by titration 170. Calc. for $C_8H_8O_4$: C, 57·2; H, 4·8%; equiv., titrating as a monobasic acid, 168.) An aqueous solution of the acid gives no colour with $FeCl_3$.

The above acid (0·38 g.) was esterified with an excess of diazomethane in ether in the usual way. The resultant light-yellow solid (0·42 g.) was crystallized first from ethyl acetate-light petroleum b.p. 60–80° giving colourless needles (0·22 g.), m.p. 187–189°. Recrystallization from benzene gave colourless needles of pure methyl 3:5-dihydroxy-*p*-toluate (0·11 g.), m.p. 189–190°, not depressed on admixture with an authentic synthetic specimen, m.p. 189–190°. (Found: C, 59·2, 59·4; H, 5·5, 5·5; OCH_3, 17·0. Calc. for $C_9H_{10}O_4$: C, 59·3; H, 5·5; $1OCH_3$, 17·0%.) An aqueous solution of the ester gives a lavender colour with $FeCl_3$.

The above methyl 3:5-dihydroxy-*p*-toluate (125 mg.) was acetylated in the usual way with anhydrous sodium acetate (250 mg.) and acetic anhydride (1 ml.). The acetylation product was purified by crystallization from methanol (+charcoal) and then from light petroleum b.p. 60–80°. *Methyl* 3:5-*diacetoxy-p-toluate* was thus obtained as colourless shining needles (63 mg.), m.p. 101–101·5° not depressed on admixture with an authentic synthetic specimen, m.p. 101–101·5°. (Found: C, 59·0, 58·7; H, 5·4, 5·3; OCH_3, 11·6. $C_{12}H_{14}O_6$ requires C, 58·7; H, 5·3; $1OCH_3$, 11·7%.)

C. iso*Cyclopaldic acid*

Action of KOH *at 150° on cyclopolic acid. Isolation of* iso*cyclopaldic acid*

A mixture of cyclopolic acid (0·40 g.), KOH (2 g.) and water (2 ml.), contained in a nickel crucible, was held at 150° in a metal bath. Test samples of the melt were periodically acidified and treated with Brady's reagent until a precipitate ceased to be formed (15 min.). The melt was then cooled, dissolved in water (15 ml.) and acidified with HCl. The resulting light-brown curdy precipitate was collected, dried (0·18 g.), m.p. 190–200° (decomp.), and crystallized to constant melting point from aqueous methanol (+charcoal). iso*Cyclopaldic acid* was thus obtained as colourless needles, m.p. 223·5–224° (decomp.). (Found: loss in weight at 100°, 6·8. $C_{11}H_{10}O_6 \cdot H_2O$ requires H_2O, 7·0%; on dried sample: C, 55·7, 55·9; H, 4·4, 4·2; OCH_3, 13·0. $C_{11}H_{10}O_6$ requires C, 55·5; H, 4·2; $1OCH_3$, 13·0%.) The acid is readily soluble in ether, methanol and ethyl acetate and slightly soluble in benzene and water. Its aqueous solution gives with $FeCl_3$ a deep reddish-purple colour which is much redder in tone than that given by cyclopolic acid.

Monomethyl ether of methyl iso*cyclopaldate. iso*Cyclopaldic acid (75 mg.) was methylated in the usual way with dimethyl sulphate (0·45 ml.), acetone (7 ml.) and anhydrous K_2CO_3 (0·40 g.) for 1·5 hr. The recovered crude methylation product (64 mg.), m.p. 136–139°, was crystallized from light petroleum giving the *monomethyl ether of methyl* iso*cyclopaldate* as long colourless silky needles (38 mg.), m.p. 139–139·5° not depressed on admixture with the synthetic *methyl ester of 4-carboxy-5:7-dimethoxy-6-methylphthalide*, m.p. 139–139·5° (see p. 627). (Found: C, 58·6, 58·8; H, 5·1, 5·3; OCH_3, 34·8. $C_{12}H_{14}O_6$ requires C, 58·6; H, 5·3; $3OCH_3$, 35·0%.)

iso*Cyclopaldic acid monomethyl ether.* The monomethyl ether of methyl *iso*cyclopaldate (0·30 g.) was refluxed for 0·75 hr. with a solution of KOH in 1:1 water-ethanol (0·2N; 10 ml.). Ethanol was removed *in vacuo* at 50° and the residue was diluted with water and acidified with HCl. The resulting precipitate was collected (0·27 g.), m.p. 194·5–195·5°, and crystallized from methanol (+charcoal) giving iso*cyclopaldic acid monomethyl ether* as colourless needles, m.p. 198–199° not depressed on admixture with synthetic *4-carboxy-5:7-dimethoxy-6-methylphthalide*, m.p. 198–198·5° (see p. 627). (Found: C, 57·2, 57·2; H, 4·6, 4·7; OCH_3, 24·3. $C_{12}H_{12}O_6$ requires C, 57·2; H, 4·8; $2OCH_3$, 24·6%.) The acid titrates in the cold to phenolphthalein as a monobasic acid, in 1:1 methanol-water solution. (Found: equiv. 249. $C_{12}H_{12}O_6$, titrating as a monobasic acid, requires equiv. 252.) On heating with an excess of 0·1N-NaOH and back titration it titrates as a dibasic acid. (Found: equiv. 125. $C_{12}H_{12}O_6$, titrating as a monoacid monolactone requires equiv. 126.)

Decarboxylation of iso*cyclopaldic acid*

(*a*) *With* HI *and red* P. *Isolation of β-orcinol* (2:6-*dihydroxy*-1:4-*dimethylbenzene*). In a quantitative estimation *iso*-cyclopaldic acid (200 mg.) was refluxed with HI (*d*, 1·7; 3 ml.) and red P (0·3 g.) in a slow stream of CO_2-free N_2. The effluent gases were first scrubbed through a red P-water trap and then through bubblers containing 0·2N-Ba(OH)$_2$ which was titrated with 0·1N-HCl at intervals. CO_2 was evolved as

follows. After 0·75 hr., 0·55 mol. equiv. CO_2; 3 hr., 1·33 mol. equiv.; 7 hr., 1·89 mol. equiv.; 9 hr., 2·06 mol. equiv.

In a preparative experiment *iso*cyclopaldic acid (0·73 g.) was refluxed in N_2 for 20 hr. with HI (*d*, 1·7; 10 ml.) and red P (3 g.). P was removed by filtration, the orange filtrate was treated with aqueous NaOH (40%; 5 ml.) and the still acid solution was extracted with ethyl acetate (4 × 20 ml.). The extract was washed with water and aqueous $Na_2S_2O_3$ and dried over anhydrous Na_2SO_4. Removal of the solvent at 50° *in vacuo* yielded pale-yellow gummy needles (0·39 g.), m.p. 145–155° which were purified to constant melting point by repeated sublimation in a high vacuum and crystallization from benzene, giving finally β-orcinol (108 mg.) as colourless jagged needles, m.p. 161·5–163° not depressed on admixture with authentic synthetic β-orcinol, m.p. 161·5–162·5° (see p. 626). (Found: C, 69·7, 69·8; H, 7·2, 7·4. Calc. for $C_8H_{10}O_2$: C, 69·5; H, 7·3%.) Its aqueous solution gave a blue colour with a faint red tinge with $FeCl_3$.

β-Orcinol (50 mg.), obtained by decarboxylation of *iso*cyclopaldic acid, was acetylated at 140° in the usual way with anhydrous sodium acetate (50 mg.) and acetic anhydride (0·2 ml.). The gum obtained on addition of water was extracted with ether and the ethereal extract was washed with aqueous Na_2CO_3 and water. On removal of the solvent from the dried extract there remained a colourless gum (65 mg.) which slowly crystallized, m.p. 42–43°. Crystallization from light petroleum, b.p. 40–60°, gave β-orcinol diacetate as colourless needles, m.p. 44–45° unchanged on further recrystallization and not depressed on admixture with an authentic synthetic specimen, m.p. 44–44·5° (see p. 626). (Found: C, 65·0, 65·0; H, 6·2, 6·4. Calc. for $C_{12}H_{14}O_4$: C, 64·9; H, 6·4%.)

(*b*) *With copper chromite and quinoline. Methylation of decarboxylation product and isolation of* 5:7-*dimethoxy*-6-*methylphthalide. iso*Cyclopaldic acid (174 mg.) was heated at 180–190° with copper chromite (150 mg.) and pure redistilled quinoline (4 ml.) in a stream of CO_2-free N_2. The effluent gases were first scrubbed through 2 N-H_2SO_4 and then through 0·2 N-$Ba(OH)_2$ which was titrated with 0·1 N-HCl at intervals. CO_2 was evolved as follows. After 0·5 hr., 0·58 mol. equiv.; 2 hr., 0·76 mol. equiv.; 2·25 hr., 0·82 mol. equiv. The reaction was now stopped. 5 N-HCl (20 ml.) was added and the solution was extracted first with ether (4 × 20 ml.) and then with ethyl acetate. The extracts were washed successively with 2 N-HCl, water, aqueous $NaHCO_3$ and water. On removal of the solvent from the dried extracts there remained an oily solid (24 mg.) from ether, and a gummy solid (31 mg.) from ethyl acetate. The combined extracts, which gave a dirty brown-red colour with $FeCl_3$ in methanol, were methylated in the usual way with dimethyl sulphate (0·3 ml.), K_2CO_3 (0·4 g.) and acetone (7 ml.). A methanolic solution of the crude methylated product was left to evaporate to dryness and the resulting brown solid (40 mg.) was separated mechanically from an oily residue. The solid was purified by sublimation *in vacuo* at 140° followed by crystallization from light petroleum, b.p. 60–80°, giving 5:7-*dimethoxy*-6-*methylphthalide* as long colourless lustrous needles (22 mg.), m.p. 171·5–172·5° not depressed on admixture with an authentic synthetic specimen, m.p. 172–172·5° (see p. 627). (Found: C, 63·2; H, 5·7; OCH_3, 29·5. $C_{11}H_{12}O_4$ requires C, 63·5; H, 5·8; $2OCH_3$, 29·8%.)

(*c*) *With aqueous* KOH. *Methylation of decarboxylation product and isolation of* 5:7-*dimethoxy*-6-*methylphthalide. iso*Cyclopaldic acid (0·20 g.) was refluxed for 6·5 hr. with aqueous KOH (28% (w/v); 5 ml.). The solution was acidified with HCl and extracted with ether. The ethereal extract was washed with aqueous $NaHCO_3$ and dried over Na_2SO_4. Acidification of the $NaHCO_3$ extract gave unchanged *iso*cyclopaldic acid (100 mg.). Removal of the solvent from the ether extract yielded an oily red solid (41 mg.) which gave an intense purple colour with methanolic $FeCl_3$. The oily red solid (40 mg.) was methylated with dimethyl sulphate, K_2CO_3 and acetone and gave a small yield (2 mg.) of 5:7-*dimethoxy*-6-*methylphthalide*, m.p. 168–169° not depressed on admixture with an authentic synthetic specimen, m.p. 172–172·5° (see p. 627).

During the preparation of *iso*cyclopaldic acid from cyclopolic acid by heating with KOH at 150° (see p. 621) there accumulated a considerable quantity of acidified aqueous liquors from which the *iso*cyclopaldic acid had been removed by filtration. These liquors, on treatment as described above, gave a sodium bicarbonate-insoluble gum which gave an intense purple colour with $FeCl_3$ and on methylation gave 5:7-dimethoxy-6-methylphthalide.

Degradation of the lichen acid, barbatolic acid, to 5:7-*dimethoxy*-6-*methylphthalide*

Through the courtesy of Dr E. E. Suominen, Chemical Laboratory, University of Helsinki, Finland, we received a specimen of the reduction product, $C_{19}H_{20}O_8$, m.p. 189°, prepared by him by reduction with Zn dust and HI at 70° of the methyl ester of barbatolic acid from the lichen *Alectoria implexa* (Hoffm.) Nyl. f. *fuscidula* Arn. (Suominen, 1939). The reduction product (18 mg.) was refluxed for 4 hr. with dimethyl sulphate (0·4 ml.), dry acetone (10 ml.) and anhydrous K_2CO_3 (0·25 g.). A second portion of K_2CO_3 (0·25 g.) was added during the reaction. Acetone was then removed *in vacuo*, water (10 ml.) was added and the mixture was shaken vigorously for 0·5 hr. The methylated product was extracted with ether (3 × 10 ml.), and on removal of the ether was obtained as a pale-yellow thick gum. This crude product, without further purification, was refluxed for 4 hr. with ethanolic NaOH (0·2 N; 4 ml.). Water was added, the solution was acidified with HCl and the hydrolysis products were extracted with ether (4 × 10 ml.). The ethereal extract was washed successively with water, saturated $NaHCO_3$ solution and again with water. On removal of the solvent from the dried (Na_2SO_4) extract there remained colourless gummy needles (5 mg.), m.p. 156–161°. These were crystallized from aqueous methanol giving long colourless needles, m.p. 171·5°, resetting and remelting at the same temperature and not depressed on admixture with synthetic 5:7-*dimethoxy*-6-*methylphthalide*, m.p. 172–172·5° (see p. 627). The mixed melt reset and remelted at the same temperature. Suominen (1939), whose method for the preparation of this substance we followed, except that he methylated the reduction product, m.p. 189°, with diazomethane for 2 months, gives the m.p. as 173·5°.

D. $KMnO_4$ *oxidation experiments*

(*a*) *Oxidation of methyl cyclopolate monomethyl ether with cold alkaline* $KMnO_4$. *Isolation of* 4:6-*dimethoxy*-5-*methylbenzene*-1:2:3-*tricarboxylic acid* (*VIII*). The monomethyl ether of methyl cyclopolate (1·2 g.) was dissolved in cold aqueous 0·04 N-KOH (24 ml.), and aqueous $KMnO_4$ (5%; 50 ml. equiv. to 5·3 atoms O) was added. After standing at

room temperature for 24 hr. almost all the $KMnO_4$ had been utilized. A further 12 ml. of the same $KMnO_4$ solution equiv. to 1·3 atoms O were therefore added. The solution was kept for a further 18 hr., when there was much residual $KMnO_4$. The precipitated MnO_2 was separated by filtration and washed with water. The combined filtrate and washings were decolorized with Na_2SO_3 and evaporated *in vacuo* to 15 ml. The concentrate was acidified with HCl and extracted with ethyl acetate (5 × 20 ml.). Removal of the solvent from the washed and dried (Na_2SO_4) extracts yielded a colourless gum (0·55 g.). The gum was refluxed with ether (10 ml.) giving a colourless solid which was washed with cold ether (10 ml.). The solid residue (0·23 g.), m.p. 185–190°, was collected and was purified by crystallization from ether giving colourless compact prisms (0·13 g.). (Found, on material dried to constant weight at 100° *in vacuo*: C, 54·3, 54·2; H, 3·9, 4·0; OCH_3, 23·4; equiv. by titration 88. $C_{12}H_{10}O_7$, i.e. $C_{12}H_{12}O_8 - H_2O$, requires C, 54·1; H, 3·8; $2OCH_3$, 23·3%; equiv., titrating as a tribasic acid (mono-acid monoanhydride), 89.) 4:6-*Dimethoxy-5-methylbenzene*-1:2:3-*tricarboxylic acid* behaves in a characteristic fashion on heating, with the formation of the anhydride (IX). If placed in a bath, previously heated to 150°, it rapidly melts with vigorous effervescence (loss of water), but resets immediately and then remelts at 195°. If heated slowly from room temperature it loses its lustre, but no effervescence occurs. It melts at 198·5–199·5°, resets on cooling and remelts at 197·5–198·5°.

The acid (64 mg.) was refluxed in a stream of CO_2-free N_2 with HI (*d*, 1·7; 3 ml.) and a little red P. CO_2 was evolved as follows. After 0·75 hr., 1·77 mol. equiv.; 1·5 hr., 2·00 mol. equiv.; 2 hr., 2·11 mol. equiv. The decarboxylation product was recovered by extraction with ethyl acetate as a light-brown solid (44 mg.) which was purified by fractional sublimation in a high vacuum at 160–180° giving a colourless solid (26 mg.), m.p. 260–265°, which on crystallization from water gave 3:5-dihydroxy-*p*-toluic acid as colourless needles, m.p. 263·5–265·5° (decomp.) not depressed on admixture with an authentic synthetic specimen, m.p. 267–268° (decomp.) (see p. 626).

Methylation of this product (10 mg.) with ethereal diazomethane, followed by crystallization of the methylated product from benzene, gave colourless woolly needles (7 mg.) of methyl 3:5-dihydroxy-*p*-toluate, m.p. 187·5–188° not depressed on admixture with an authentic synthetic specimen, m.p. 189–190° (see p. 626).

(*b*) *Oxidation of methyl cyclopolate monomethyl ether with hot alkaline* $KMnO_4$. *Isolation of* 4:6-*dimethoxybenzene*-1:2:3:5-*tetracarboxylic acid* (*IV*). Powdered $KMnO_4$ (6·9 g.) was added in portions over a period of 4 hr. to a solution, heated under reflux, of the monomethyl ether of methyl cyclopolate (1·18 g.) in aqueous 0·03 N-KOH (40 ml.). The excess $KMnO_4$ was then destroyed by adding methanol (1 ml.). The MnO_2 was separated by filtration and washed thoroughly with water. The filtrate and washings were acidified to pH 2 with HCl, and $CaCl_2$ (1·5 g.) was added giving a precipitate of calcium oxalate (40 mg.) which was filtered off and discarded. Conc. HCl (3 ml.) was added to the filtrate which was extracted with ethyl acetate (7 × 40 ml.). The washed and dried (Na_2SO_4) extract was evaporated *in vacuo* giving a pale-yellow gummy solid (0·76 g.). This was crystallized from a mixture of ethyl acetate (1 vol.) and benzene (2 vol.) and gave microprisms (0·425 g.), m.p. 221·5–224·5° (decomp.). Two further crystallizations from the same solvents gave very small colourless prisms (0·25 g.), m.p. 225·5–228° (decomp.) with softening from 222°. (Found: loss in weight at 100° *in vacuo*, 5·8. $C_{12}H_{10}O_{10} \rightarrow C_{12}H_8O_9 + H_2O$ requires 5·7% H_2O; on sample dried to constant weight at 110° *in vacuo*: C, 48·5; H, 2·6; OCH_3, 20·8; equiv. by titration 74. $C_{12}H_8O_9$ requires C, 48·7; H, 2·7; $2OCH_3$, 21·0%; equiv., titrating as a tetrabasic acid (di-acid mono-anhydride) 74.) 4:6-*Dimethoxybenzene*-1:2:3:5-*tetracarboxylic acid* is readily soluble in water, ether and ethyl acetate, but is only slightly soluble in benzene. It readily loses 1 mol. water on heating at 100° *in vacuo* or at its melting point with the formation of the anhydride (V).

The acid $C_{12}H_{10}O_{10}$ (96 mg.) was methylated for 2 days with excess ethereal diazomethane. On evaporating the filtered solution to dryness there was obtained a colourless gum (114 mg.) which was very soluble in ether, methanol, benzene and $CHCl_3$ and slightly soluble in light petroleum. It could not be crystallized from any of these solvents or mixtures thereof. It was purified by rapid sublimation in a high vacuum at 180° giving a colourless distillate which set to a waxy solid (80 mg.), m.p. 44–46°. (Found: C, 52·2, 52·2; H, 5·0, 4·8; OCH_3, 49·5. $C_{16}H_{18}O_{10}$ requires C, 51·9; H, 4·9; $6OCH_3$, 50·3%.)

The acid $C_{12}H_{10}O_{10}$ (92·5 mg.) was refluxed in a stream of CO_2-free N_2 with HI (*d*, 1·7; 3 ml.) and a little red P. CO_2 was evolved as follows. After 0·5 hr., 2·04 mol. equiv.; 0·75 hr., 2·61 mol. equiv.; 1·25 hr., 3·03 mol. equiv.; 1·5 hr., 3·13 mol. equiv. The decarboxylation product was recovered by extraction with ethyl acetate (4 × 12 ml.) as a slightly yellow solid (45 mg.) which was purified by sublimation in a high vacuum at 190–200° giving α-resorcylic acid (3:5-dihydroxybenzoic acid) (33 mg.), m.p. 234·5–235·5° (decomp.) not depressed on admixture with an authentic synthetic specimen, m.p. 234–236° (decomp.). The above decarboxylation product (30 mg.), m.p. 234·5–235·5° (decomp.), was methylated in the usual way with dimethyl sulphate (0·2 ml.), K_2CO_3 (0·30 g.) and acetone (7 ml.). The solvent was removed by evaporation and the residue was refluxed for 1 hr. with aqueous 0·1 N-NaOH (6 ml.). The solution was acidified with HCl and extracted with ether. Removal of the solvent gave a solid (25 mg.), m.p. 178–181°, which was purified by crystallization from aqueous methanol and sublimation in a high vacuum at 120°, giving 3:5-dimethoxybenzoic acid as colourless needles (15 mg.), m.p. 182·5–183·5° not depressed on admixture with an authentic synthetic specimen m.p. 182–183°. (Found: C, 59·0; H, 5·3; OCH_3, 33·7. Calc. for $C_9H_{10}O_4$: C, 59·3; H, 5·5; $2OCH_3$, 34·1%.)

(*c*) *Oxidation of cyclopolide monomethyl ether with cold alkaline* $KMnO_4$. *Isolation of* 4-*carboxy*-5:7-*dimethoxy*-6-*methylphthalide*. A suspension of cyclopolide monomethyl ether (0·24 g.) in aqueous N-KOH (5 ml.) and aqueous $KMnO_4$ (2% (w/v); 21 ml. equiv. to 3·9 atoms O) was held at room temperature for 24 hr. with occasional shaking. MnO_2 and excess $KMnO_4$ were removed by addition of Na_2SO_3. The clear liquid was evaporated *in vacuo* to 10 ml., acidified with HCl and extracted with ether (4 × 25 ml.). Removal of the solvent gave colourless gummy needles (195 mg.) which were washed with a little ether leaving a solid residue (71 mg.), m.p. 192–195°. This was recrystallized from water giving 4-*carboxy*-5:7-*dimethoxy*-6-*methylphthalide* as fine colourless needles, m.p. 198–199° not depressed on admixture with an authentic synthetic specimen, m.p. 198–198·5° (see p. 627).

Cyclopaldic acid

Purification. Crude cyclopaldic acid was recrystallized from boiling water (300 ml./g. + charcoal). Yield about 55%, some cyclopolic acid being isolated from the mother liquors. Repeated crystallization from water gave a constant m.p. of 224–225°, but for analysis a pure sample was more readily obtained by sublimation in a high vacuum at 170°.

General properties. Cyclopaldic acid crystallizes from water in long fluffy colourless needles, m.p. 224–225° with sublimation, resetting on cooling and remelting at 222·5–224·5°. It sublimes readily in a high vacuum at 170°. (Found, on a sublimed sample: C, 55·3; H, 4·45; OCH_3, 13·1; *C*-CH_3, 5·4; equiv. by conductometric titration, 118. $C_{11}H_{10}O_6$ requires C, 55·45; H, 4·2; $1OCH_3$, 13·0; $1C$-CH_3, 6·3%; equiv. titrating as a dibasic acid, 119.) It is moderately soluble in ethanol, methanol and ethyl acetate; sparingly soluble in ether, chloroform and hot benzene and very slightly soluble in boiling light petroleum, b.p. 80–100°. It dissolves slowly with slow effervescence in cold aqueous $NaHCO_3$ to give a yellow solution. Its solution in aqueous NaOH is also yellow, the colour deepening on warming, but reverting to its original intensity on recooling. Its aqueous or ethanolic solution gives a deep ruby-red colour with $FeCl_3$, with no suggestion of blue. Brady's reagent quickly gives a gelatinous orange precipitate, which on crystallization from pyridine forms orange needles, m.p. 231–232° (decomp.). Fehling's solution is not reduced even on boiling, though its original blue colour changes to green. Bromine water, when added to a dilute aqueous solution of cyclopaldic acid, gives with the first few drops a buff colour, which on further careful addition is discharged leaving a colourless solution. No precipitate is formed even after adding excess bromine water. When a trace of cyclopaldic acid is exposed in the neck of a test tube containing aqueous NH_3 (*d*, 0·880) it rapidly turns brown and if then dissolved in the aqueous NH_3 it gives a purple colour, fading after 2–3 hr. to orange with a yellow fluorescence (cf. gladiolic acid, Raistrick & Ross, 1952; Grove, 1952). By contrast, if aqueous NH_3 (*d*, 0·880) is added rapidly to solid cyclopaldic acid no colour develops. Its methanolic solution exhibits in ultraviolet light a green fluorescence which is not shown by cyclopolic acid. It gives a pale, quickly fading, yellow colour with bleaching powder. When dissolved in buffer solution, pH 9·2, and treated with a few drops of an aqueous suspension of 2:6-dichloroquinone chloroimide a green colour is formed in 2 min., changing to blue-green after 10 min. and to dark reddish brown after 3 days.

Acetylation of cyclopaldic acid. (*a*) A mixture of cyclopaldic acid (0·20 g.), anhydrous sodium acetate (0·4 g.) and acetic anhydride (1 ml.) was heated for 0·5 hr. in an oil bath held at 150°. Addition of water, after cooling, gave an oil which quickly solidified (0·31 g.). Two crystallizations from aqueous ethanol (50% by vol.) + charcoal gave colourless needles (0·17 g.), m.p. 159°, resetting on cooling and remelting at 158°. (Found: C, 54·0; H, 5·1; OCH_3, 7·1; $COCH_3$, 38·4. $C_{19}H_{20}O_{11}$, i.e. tetra-acetyl derivative of $C_{11}H_{10}O_6$, H_2O, requires C, 53·8; H, 4·75; $1OCH_3$, 7·3; $4COCH_3$, 40·6%.) The *tetra-acetyl derivative* (XXIII) is insoluble in cold aqueous 2N-NaOH and gives no colour with $FeCl_3$ in ethanol or after dilution with water. A yellow precipitate is slowly formed on the addition of Brady's reagent to its ethanolic solution.

(*b*) A mixture of cyclopaldic acid (0·50 g.), anhydrous sodium acetate (0·50 g.) and acetic anhydride (2·5 ml.) was refluxed vigorously for 6·5 hr. in a bath held at 180–190°. The cooled dark-brown solution was treated with water (10 ml.), giving a semi-solid product which was separated and washed well with cold ethanol (8 ml.). The residual brown powder (0·33 g.), m.p. 170–195°, was crystallized from methanol (25 ml.) giving brown crystals (0·24 g.), m.p. 196–198°, which were purified for analysis by further crystallization from methanol (25 ml.), followed by sublimation in a high vacuum at 170–185° and crystallization of the sublimate (0·17 g.) from methanol. The *condensation product* (XXIV*b*) was obtained as almost colourless needles (0·16 g.) m.p. 197·5–198·5°. (Found, on two different samples: C, 58·9, 58·9; H, 4·2, 4·5; OCH_3, 10·5, 10·3. $C_{15}H_{12}O_7$ requires C, 59·2; H, 4·0; $1OCH_3$, 10·2%.) The product dissolves slowly in cold 0·1N-NaOH, more rapidly in 2N-NaOH, or on warming, to give a yellow solution. It gives no colour in ethanolic solution with $FeCl_3$ and no precipitate with Brady's reagent in aqueous ethanol, except on long standing (perhaps as a result of hydrolysis).

The condensation product (0·1293 g.) was hydrolysed by boiling for 5 hr. with N-H_2SO_4 (20 ml.). (Found, by distillation of acetic acid: $COCH_3$, 12·5. $C_{15}H_{12}O_7$ requires $1COCH_3$, 14·1%.) The insoluble colourless solid (0·109 g.), m.p. 268–274°, remaining after the distillation of the acetic acid was collected and purified by fractional sublimation in a high vacuum. The main fraction subliming at 170–200° melted at 275–277° unchanged on crystallization from ethanol. (Found: C, 59·2; H, 3·9; OCH_3, 11·8. $C_{13}H_{10}O_6$ requires C, 59·55; H, 3·8; $1OCH_3$, 11·8%.) The product, *5-formyl-7-methoxy-8-methylcoumarin-6-carboxylic acid* (XXIV*a*) dissolves slowly in $NaHCO_3$ to give a colourless solution, but at once in 2N-Na_2CO_3 or 2N-NaOH to give a yellow solution. It gives no colour with ethanolic $FeCl_3$. Its *2:4-dinitrophenylhydrazone* was prepared by the addition of Brady's reagent (34 ml.) to a solution of it (21 mg.) in ethanol (21 ml.) giving, after 24 hr., yellow needles (22 mg.), m.p. 230–232° raised to 256–257° with effervescence, after two crystallizations from ethanol. (Found: C, 49·6; H, 3·8; N, 11·9. $C_{19}H_{14}O_9N_4$, H_2O requires C, 49·6; H, 3·5; N, 12·2%.) The compound gives a red Neuberg reaction; it is completely extracted from an ethereal solution by aqueous $NaHCO_3$.

Reaction and degradation products of cyclopaldic acid

(*a*) *Reaction of cyclopaldic acid with hydrazine. Formation of 5-hydroxy-7-methoxy-6-methylphthalazine (XXV)*

Cyclopaldic acid (0·24 g.) was treated with hydrazine hydrate (50%; 0·10 ml.). Water (3 ml.) was added and the whole was boiled for 15 min. The supernatant liquid now no longer gave a precipitate with Brady's reagent. The pale-lime coloured rods (0·144 g.), m.p. 260–262°, which separated, were collected and crystallized from ethanol (40 ml.) giving the *condensation product* as very pale-yellow prisms, m.p. 260–262° (decomp.) with preliminary darkening. (Found: C, 63·1; H, 5·45; N, 14·9; OCH_3, 16·3. $C_{10}H_{10}O_3N_2$ requires C, 63·1; H, 5·3; N, 14·7; $1OCH_3$, 16·3%.) *5-Hydroxy-7-methoxy-6-methylphthalazine* is insoluble in cold water or in freshly prepared aqueous 2% $NaHCO_3$, but dissolves slightly in hot water to give a yellow solution. It dissolves in 2N-Na_2CO_3 or in 2N-NaOH to give a nearly colourless

solution. Brady's reagent gives no precipitate in aqueous solution. $FeCl_3$ gives an orange-red colour in ethanolic solution changing to pale brown on addition of water. The *hydrochloride* was prepared by dissolving the pure phthalazine (28 mg.) in warm 2N-HCl (3 ml.), filtering and allowing to cool to room temperature. The long needles (34 mg.), m.p. 248–250° (decomp.), which had a slight pink colour in bulk, were filtered, washed with 2N-HCl and dried in a high vacuum. (Found: C, 52·8; H, 5·0; N, 12·4; Cl, 16·0; OCH_3, 12·9. $C_{10}H_{10}O_2N_2$, HCl requires C, 53·0; H, 4·9; N, 12·4; Cl, 15·7; $1OCH_3$, 13·7%.)

In a repeat experiment, a mixture of cyclopaldic acid (0·121 g.), hydrazine hydrate (50%; 0·05 ml.) and water (1·5 ml.) was boiled for 15 min. CO_2, estimated by absorption in 0·2N-$Ba(OH)_2$, amounting to 66% of 1 mol. CO_2 was evolved, and 5-hydroxy-7-methoxy-6-methylphthalazine (80 mg.), m.p. 258–261° (decomp.), was collected. Recrystallization from ethanol (25 ml.) gave the pure base (47 mg.), m.p. 261–262° (decomp.).

(*b*) *Action of boiling aqueous* 2N-NaOH *on cyclopaldic acid. Isolation of* iso*cyclopaldic acid* (*XVII*; R = R_1 = H)

A solution of cyclopaldic acid (0·112 g.) in aqueous 2N-NaOH (10 ml.) was refluxed for 15 min. The solution was acidified to congo red with 2N-HCl. *iso*Cyclopaldic acid was precipitated as colourless needles (0·065 g.), m.p. 217° (decomp.), which were collected and crystallized from 50% aqueous methanol (7 ml.). The slightly pink needles (53 mg.) were sublimed at 130–140° in a high vacuum to yield a colourless sublimate, m.p. 224–225°, with sublimation and slight effervescence not depressed on admixture with *iso*cyclopaldic acid, m.p. 223·5–224°, prepared from cyclopolic acid by heating with KOH at 150° (see p. 621). A mixture with cyclopaldic acid, m.p. 224–225°, melted at 200–204°. (Found: C, 55·5; H, 4·5. $C_{11}H_{10}O_6$ requires C, 55·5; H, 4·2%.) It dissolved in aqueous $NaHCO_3$ with effervescence to a colourless solution. It gave no yellow precipitate with Brady's reagent. Its aqueous solution gave with $FeCl_3$ a deep reddish-purple colour which is much bluer in tone than that given by cyclopaldic acid and much redder than that given by cyclopolic acid.

(*c*) *Oxidation of cyclopaldic acid with alkaline* H_2O_2

Isolation of oxycyclopaldic acid (6-*formyl*-5-*hydroxy*-3-*methoxy*-4-*methylphthalic acid* (*XXVI*). *Its derivatives and oxidation products.* H_2O_2 ('100 vol.'; 2 ml.) was added to a solution of cyclopaldic acid (0·4 g.) in aqueous 2N-NaOH (40 ml.). After standing at room temperature for 10 min. the solution was added rapidly to 2N-HCl (42 ml.), cooled and extracted at once with ethyl acetate (2 × 80 ml.). The extract was washed with water (5 ml.) and on removal of the solvent there remained a dry, nearly colourless solid (0·38 g.). This was crystallized from ethyl acetate (5 ml.) and benzene (50 ml.) giving colourless needles (0·28 g.), m.p. 136–137°, with slight effervescence. Recrystallization from the same solvents gave pure *oxycyclopaldic acid*, m.p. 138°, with slight effervescence; water is apparently evolved and the melt resets on cooling to remelt at 140–142° without decomposition. (Found: C, 51·8; H, 3·8; OCH_3, 12·3. $C_{11}H_{10}O_7$ requires C, 52·0; H, 4·0; $1OCH_3$, 12·2%.) Oxycyclopaldic acid is soluble in water. The colourless aqueous solution turns yellow on the addition of NaOH, gives a purplish-red colour with $FeCl_3$ and an immediate yellow precipitate with Brady's reagent.

Oxycyclopaldic anhydride. Oxycyclopaldic acid (35 mg.) was heated to 160° in a test tube for 5 min., cooled and sublimed at 100–125° in a high vacuum. The pale-yellow sublimate, m.p. 141–142°, was recrystallized from light petroleum (5 ml., b.p. 80–100°). *Oxycyclopaldic anhydride* (22 mg.) separated as long pale-yellow needles, m.p. 141° with sublimation. (Found: C, 55·9; H, 3·5; OCH_3, 13·2 $C_{11}H_8O_6$ requires C, 55·9; H, 3·4; $1OCH_3$, 13·1%.)

Unlike cyclopaldic acid, cyclopolic acid is not oxidized with alkaline H_2O_2. A solution of cyclopolic acid (0·25 g.) in water (25 ml.) was treated with 2N-NaOH (5 ml.) and H_2O_2 ('100 vol.'; 3 ml.), and was held at room temperature for 3·5 hr. The solution was acidified with HCl and extracted with ether (3 × 20 ml.). On removal of the solvent, unchanged cyclopolic acid (0·20 g.), m.p. 142° (decomp.), remained. It was identified by recrystallization, m.p. 146·5° (decomp.) not depressed on admixture with cyclopolic acid.

Monomethyl oxycyclopaldate methyl ether (*XXVII*) (*methyl*-(2) 6-*formyl*-3:5-*dimethoxy*-4-*methylphthalate*). A mixture of oxycyclopaldic acid (0·10 g.), dimethyl sulphate (1·0 ml.), anhydrous K_2CO_3 (1·0 g.) and dry acetone (20 ml.) was refluxed for 1·5 hr. The mixture, initially colourless, soon turned yellow, then paled gradually until, after 0·5 hr., it became nearly colourless again. Acetone was removed under reduced pressure and the residue was shaken vigorously with water (20 ml.) until all the oil had dissolved. The yellow solution was extracted twice with ethyl acetate to remove neutral impurities, then acidified to congo red and re-extracted twice with ethyl acetate. On removal of the solvent there remained a gummy solid (0·10 g.) which was crystallized twice from water (second time + charcoal) giving *monomethyl oxycyclopaldate methyl ether* (0·035 g.) as colourless rhombic plates which appeared to have a double m.p., 107–109° or 125–127°, and after cooling will remelt at either of these temperatures or sometimes intermediately. (Found: C, 55·4; H, 5·0; OCH_3, 32·8. $C_{13}H_{14}O_7$ requires C, 55·3; H, 5·0; $3OCH_3$, 33·0%.) This mono-ester is soluble in aqueous $NaHCO_3$, gives no colour with NaOH or $FeCl_3$, but gives a yellow crystalline precipitate with Brady's reagent.

Oxidation of monomethyl oxycyclopaldate methyl ether with $KMnO_4$. (i) *Mild oxidation.* A solution of monomethyl oxycyclopaldate methyl ether (0·20 g.) and $NaHCO_3$ (1 g.) in water (20 ml.) was treated with aqueous 0·05M-$KMnO_4$ (20 ml.) and boiled. The purple colour gradually disappeared and further 10 ml. portions of $KMnO_4$ were added until the purple colour remained for 5 min. when a total of 75 ml. had been added. The mixture was cooled, decolorized with $NaHSO_3$, acidified to congo red and extracted with ethyl acetate (2 × 100 ml.). The extracts were washed with a little water (10 ml.) and evaporated. The residual gum (0·23 g.) was crystallized from ethyl acetate (2 ml.) and benzene (25 ml.), adding light petroleum (15 ml., b.p. 60–80°) to the filtrate.

3-*Carbomethoxy*-4:6-*dimethoxy*-5-*methylphthalic acid* (0·142 g.) separated as colourless rosettes of plates, m.p. 135–137°, with gentle effervescence and loss of water; resetting with difficulty on cooling and remelting at 90–100°. Recrystallization did not raise the melting point. (Found: C, 52·4; H, 4·6; OCH_3, 30·7. $C_{13}H_{14}O_8$ requires C, 52·35;

H, 4·7; $3OCH_3$, 31·2%.) This mono-ester does not give a colour with $FeCl_3$ or a precipitate with Brady's reagent. The same product is also obtained when the oxidation is carried out with cold $KMnO_4$ in dilute NaOH solution.

3-Carbomethoxy-4:6-dimethoxy-5-methylphthalic anhydride. The above mono-ester (50 mg.) was heated at 150° for 1 hr. The melt was cooled and crystallized from light petroleum (5 ml., b.p. 60–80°) + charcoal. The *anhydride* (22 mg.) separated as colourless fluffy needles, m.p. 90° or 101–103°, resetting on cooling and remelting at one or other of these temps. (Found: C, 56·1, 56·1; H, 4·5, 4·3; OCH_3, 33·3. $C_{13}H_{12}O_7$ requires C, 55·7; H, 4·3; $3OCH_3$, 33·2%.) The anhydride does not dissolve in aqueous $NaHCO_3$.

Hydrolysis of the mono-ester. The mono-ester (50 mg.) was hydrolysed by boiling with aqueous 2N-NaOH (10 ml.) for 1 hr. The solution was cooled, acidified and extracted with ethyl acetate (2 × 50 ml.). The extracts were washed with water (5 ml.) and evaporated. The residue (30 mg.) was crystallized from ethyl acetate (3 ml.) and benzene (30 ml.). Fine colourless needles (25 mg.) separated, m.p. 198–199°, not depressed on admixture with 4:6-dimethoxy-5-methylbenzene-1:2:3-tricarboxylic acid (VIII), m.p. 198·5–199·5°, obtained by oxidation of methyl cyclopolate monomethyl ether with cold alkaline $KMnO_4$ (see p. 622).

(ii) *Vigorous oxidation.* Monomethyl oxycyclopaldate methyl ether (0·22 g.) was oxidized with hot alkaline $KMnO_4$ under the same conditions as for the oxidation of methyl cyclopolate monomethyl ether with the same reagent (see p. 623, § *b*). The oxidation product obtained (48 mg.), m.p. 224–226·5° (decomp.), did not depress the melting point of the product, 4:6-dimethoxybenzene-1:2:3:5-tetracarboxylic acid, obtained by oxidation of methyl cyclopolate monomethyl ether.

Syntheses

β-Orcinol (2:6-*dihydroxy*-1:4-*dimethylbenzene* (*XVIII*). β-Orcinol was synthesized by the method of Sonn (1931). By crystallization from benzene it was obtained as colourless jagged needles, m.p. 161·5–162·5°. (Sonn records the m.p. as 163°.) (Found: C, 69·2; H, 7·3. Calc. for $C_8H_{10}O_2$: C, 69·5; H, 7·3%.) An aqueous solution of β-orcinol gives a blue colour with a faint red tinge with $FeCl_3$.

β-Orcinol diacetate. Synthetic β-orcinol (30 mg.) was heated at 140° for 0·75 hr. with anhydrous sodium acetate (50 mg.) and acetic anhydride (0·2 ml.). Addition of water precipitated an oil which was extracted with ether. The ethereal extract was washed with 2N-Na_2CO_3 and water. Removal of the solvent from the dried (Na_2SO_4) extract gave a colourless gum (42 mg.) which slowly crystallized on rubbing, m.p. 43·5–44°. It was purified by crystallization from light petroleum, b.p. 40–60°. β-Orcinol diacetate was obtained as thick colourless needles (21 mg.), m.p. 44–44·5° unchanged by recrystallization from ethanol. (Found: C, 64·7; H, 6·4. Calc. for $C_{12}H_{14}O_4$: C, 64·9; H, 6·4%.) The only melting point previously recorded for this substance was 69° by Herzig & Wenzel (1906).

3:5-Dihydroxy-p-toluic acid (*3:5-dihydroxy-4-methylbenzoic acid*) (*X*). *p*-Toluic acid was sulphonated according to the method of Asahina & Asano (1933). The Ba salt of the resulting disulphonic acid was isolated as colourless needles. (Found, on three different preparations dried to constant weight at 100° *in vacuo*: Ba, 32·0, 31·1, 30·8. Calc. for $C_8H_6O_8S_2Ba$: Ba, 31·8%.) The Ba salt was converted into the K salt by interaction with K_2CO_3 in aqueous solution. The K salt of the disulphonic acid was converted into 3:5-dihydroxy-*p*-toluic acid by fusion with KOH essentially by the method of Charlesworth & Robinson (1934). It was purified by repeated crystallization from water giving colourless needles, m.p. 267–268° (decomp.). (Asahina & Asano give m.p. 262°, decomp.)

The acid was treated with excess ethereal diazomethane and the methylation product was crystallized from benzene giving methyl 3:5-dihydroxy-*p*-toluate as colourless needles, m.p. 189–190°. (Asahina & Hayashi (1933) give m.p. 190°.) On acetylation of this ester with acetic anhydride and sodium acetate in the usual way and crystallization of the recovered acetate from light petroleum, b.p. 60–80°, *methyl 3:5-diacetoxy-p-toluate* was obtained as colourless needles, m.p. 101–101·5°. (Found: C, 59·1; H, 5·3; OCH_3, 12·0. $C_{13}H_{14}O_6$ requires C, 58·7; H, 5·3; $1OCH_3$, 11·7%.)

3:5-Dihydroxy-4-methylbenzyl alcohol. Excess lithium-aluminium hydride (6·0 g.) was refluxed for 3 hr. with ether (170 ml.) previously dried over Na-K liquid alloy, the solid hydride being crushed occasionally with a glass rod to facilitate solution. The resulting cloudy solution was vigorously stirred and 3:5-dihydroxy-*p*-toluic acid (4·7 g.), m.p. 267–268°, previously dried to constant weight at 100° *in vacuo* and dissolved in a little dry ether, was added dropwise at a rate sufficient to produce a gentle reflux. The thick suspension was stirred and refluxed for a further 3 hr. It was then cooled in an ice bath with constant stirring, and water followed by 2N-H_2SO_4 (250 ml.) was cautiously added. The clear aqueous layer was saturated with NaCl, the ether layer was separated and the aqueous layer was re-extracted with ether (4 × 100 ml.). Removal of the solvent from the combined dried (Na_2SO_4) ether extracts gave light brown slightly resinous needles (3·7 g.), m.p. 145–160°. By cautious washing with a little ether much of the gum was removed and the resulting solid (3·0 g.) melted at 154–156·5°, yield 72%. On crystallization from ethyl acetate-benzene-light petroleum it gave *3:5-dihydroxy-4-methylbenzyl alcohol* as colourless lustrous plates, m.p. 163–163·5°. (Found: C, 62·0; H, 6·4. $C_8H_{10}O_3$ requires C, 62·3; H, 6·5%.) 3:5-Dihydroxy-4-methylbenzyl alcohol is very soluble in water, and its aqueous solution gives a purple-blue colour with $FeCl_3$. With aqueous sodium hypochlorite it gives an intense maroon-red colour which fades only very slowly on standing.

3:5-Diacetoxy-4-methylbenzyl acetate. 3:5-Dihydroxy-4-methylbenzyl alcohol (25 mg.) was acetylated in the usual way at 140° with sodium acetate (40 mg.) and acetic anhydride (0·1 ml.). The crude acetate (43 mg.), m.p. 53·5–55°, recovered on addition of water, on crystallization from light petroleum gave *3:5-diacetoxy-4-methylbenzyl acetate* as thick colourless needles (25 mg.), m.p. 60–60·5°. (Found: C, 59·9; H, 6·1. $C_{14}H_{16}O_6$ requires C, 60·0; H, 5·8%.)

Tri-p-nitrobenzoate of 3:5-dihydroxy-4-methylbenzyl alcohol. A solution of 3:5-dihydroxy-4-methylbenzyl alcohol (15 mg.) in pyridine (0·25 ml.) was refluxed with *p*-nitrobenzoyl chloride (58 mg., 3·2 mol.) at 120° for 20 min. On cooling, colourless needles separated. Water was added and the product was filtered off and washed with water. It was then triturated with 0·5N-NaOH (5 ml.), separated by filtration, washed and dried giving colourless needles (45 mg.), m.p. 254·5–255°. Crystallization from pyridine gave the *tri-p-nitrobenzoate of 3:5-dihydroxy-4-methylbenzyl alcohol* as small colourless needles, m.p. 255·5–256°, resetting and remelting at 254°. (Found: C, 58·1; H, 3·2; N, 7·2. $C_{29}H_{19}O_{12}N_3$ requires C, 57·9; H, 3·2; N, 7·0%.) It is soluble

in acetic acid and pyridine but only slightly soluble in methanol and ethyl acetate.

Di- and mono-carboxylation of 3:5-dihydroxy-4-methylbenzyl alcohol. CO_2 was bubbled through a mixture of 3:5-dihydroxy-4-methylbenzyl alcohol (0·91 g.), glycerol (8·5 ml.) and $KHCO_3$ (6 g.) which was heated under reflux at 150° for 3 hr. After cooling, water (80 ml.) was added and the red-brown solution was saturated with NaCl and extracted with ether (3 × 50 ml.). Removal of the solvent gave neutral, brown, slightly gummy, jagged needles (0·33 g.), m.p. 131–148°, consisting essentially of unchanged 3:5-dihydroxy-4-methylbenzyl alcohol.

4-Carboxy-5:7-dihydroxy-6-methylphthalide. The alkaline ether-extracted aqueous layer was acidified with HCl and re-extracted with ether (3 × 50 ml.). Removal of the solvent gave a dark-brown gummy solid (0·60 g.) which was dissolved in a small volume of methanol and held at 0° for 3 days. A brown solid (80 mg.), m.p. 261·5° (decomp.), separated and was collected. (For treatment of the mother liquors see below.) The solid was purified in the following steps; crystallized from methanol, plates and thick needles (55 mg.), m.p. 265°; sublimed in a high vacuum at 180–190°, small colourless needles (50 mg.), m.p. 276·5–277·5° (decomp.), completely soluble in $NaHCO_3$; crystallized from methanol, plates and a few thick needles, m.p. 265° (decomp.), crystallized from acetone, plates (30 mg.), m.p. 260–261° (decomp.). Further crystallization from acetone effected no change in melting point. When acetone alone was used for the purification of the crude acid a similar initial rise in melting point occurred, followed by a fall to constant melting point of 260–261° with some effervescence and decomposition. (Found, on sample dried at 100° *in vacuo* C, 53·5; H, 3·9. $C_{10}H_8O_6$ requires C, 53·6; H, 3·6%.) This product, *4-carboxy-5:7-dihydroxy-6-methylphthalide*, dissolves with effervescence in $NaHCO_3$ and colourless needles of the sodium salt readily separate from the solution on standing. An ethanolic solution of the free acid gives an intense red-purple colour with $FeCl_3$, redder in tone than that given by salicylic acid.

Methyl ester of 4-carboxy-5:7-dimethoxy-6-methylphthalide (*XVII*; R = R_1 = CH_3). 4-Carboxy-5:7-dihydroxy-6-methylphthalide (35 mg.) was heated under reflux for 1·5 hr. with dimethyl sulphate (0·2 ml.), K_2CO_3 (0·30 g.) and acetone (10 ml.). Evaporation of the solvent and addition of water gave the crude product (27 mg.), m.p. 131–132°, which was purified by sublimation in a high vacuum at 120° giving the *methyl ester of 4-carboxy-5:7-dimethoxy-6-methylphthalide* as colourless fluffy needles (20 mg.), m.p. 139–139·5° resetting on cooling and remelting at 138·5–139°. The melting point was unchanged by crystallization from light petroleum and was not depressed on admixture with the monomethyl ether of methyl *iso*cyclopaldate (see p. 621). (Found: C, 58·6; H, 5·2; OCH_3, 34·7. $C_{12}H_{14}O_6$ requires C, 58·6; H, 5·3; $3OCH_3$, 35·0%.)

4-Carboxy-5:7-dimethoxy-6-methylphthalide (*XVII*; R = CH_3, R_1 = H). The above methyl ester (25 mg.) was refluxed for 0·5 hr. with aqueous 0·5 N-NaOH (5 ml.). Acidification with HCl gave colourless needles (21 mg.), m.p. 196–197°. On crystallization from aqueous methanol, *4-carboxy-5:7-dimethoxy-6-methylphthalide* was obtained as colourless silky needles (12 mg.), m.p. 198–198·5° not depressed on admixture with *iso*cyclopaldic acid monomethyl ether (see p. 621). (Found: C, 57·6; H, 5·1; OCH_3, 24·5. $C_{12}H_{12}O_6$ requires C, 57·2; H, 4·8; $2OCH_3$, 24·6%.)

5:7-Dimethoxy-6-methylphthalide (*XIX*). The methanolic mother liquors (see above), from which the bulk of the 4-carboxy-5:7-dihydroxy-6-methylphthalide had been removed, gave on evaporation a little solid and mainly a gum (0·50 g.) which was extracted with cold water (10 ml.). The undissolved brown solid (52 mg.) was separated by filtration and consisted of crude 4-carboxy-5:7-dihydroxy-6-methylphthalide. The aqueous filtrate was acidified with HCl, boiled for 10 min. and the resulting dark-brown deposit (25 mg.) was filtered off and discarded. The acidified filtrate was saturated with NaCl and extracted with ether (3 × 25 ml.). Removal of the solvent gave a soft amber gum (0·35 g.) which was sublimed in a high vacuum at 190–195° giving a slightly gummy sublimate (0·10 g.) still containing 4-carboxy-5:7-dihydroxy-6-methylphthalide which could not be separated by fractional crystallization or fractional sublimation. The gummy sublimate (100 mg.) was therefore methylated with dimethyl sulphate (0·3 ml.), K_2CO_3 (0·3 g.) and acetone (10 ml.). The solvent was removed and the residue was refluxed for 0·5 hr. with 2 N-NaOH (10 ml.). Neutral material was removed by extraction with ether and the alkaline liquors were acidified with HCl. The resulting brown precipitate was filtered off and dissolved in ethyl acetate. The ethyl acetate solution was thoroughly extracted with $NaHCO_3$ to remove 4-carboxy-5:7-dimethoxy-6-methylphthalide. Evaporation of the dried ethyl acetate solution gave brown needles (42 mg.) which were purified by sublimation at 125° in a high vacuum giving slightly yellow needles (31 mg.), m.p. 169·5–170°. Crystallization from light petroleum gave *5:7-dimethoxy-6-methylphthalide* as thin colourless lustrous needles (23 mg.), m.p. 172–172·5° not depressed on admixture with the methylated product from the decarboxylation of *iso*cyclopaldic acid with copper chromite and quinoline (see p. 622). (Found: C, 63·6; H, 5·7; OCH_3, 29·5. $C_{11}H_{12}O_4$ requires C, 63·5; H, 5·8; $2OCH_3$, 29·8%.)

5:7-Dimethoxy-4:6-dimethylphthalide (*XIV*). A solution of 5:7-dimethoxy-6-methylphthalide (41 mg.) in glacial acetic acid (0·2 ml.) and chloromethyl methyl ether (0·2 ml.) was heated for 20 hr. at 90–95°. The solvents were removed *in vacuo* over NaOH. The light-brown gummy residue was dissolved in methanol (7 ml.) and dechlorinated by shaking with Adams's catalyst (40 mg.) in an atmosphere of H_2 for 4 hr. The catalyst was removed by filtration. On removal of the solvent there remained a colourless gum which partly crystallized in needles on standing. It consisted of a mixture of the reaction product and unchanged starting material. The mixture was fractionated by alternate fractional sublimation in a high vacuum and crystallization of the sublimate from light petroleum, giving finally *5:7-dimethoxy-4:6-dimethylphthalide* as colourless needles (1·5 mg.), m.p. 98–100° not depressed on admixture with the methylated Clemmensen's reduction product from cyclopolic acid, m.p. 101·5–102° (see p. 621).

SUMMARY

1. Two hitherto undescribed mould metabolic products, *cyclopolic acid* and *cyclopaldic acid*, have been isolated from culture filtrates of each of two different strains of *Penicillium cyclopium* Westling, one of which is a typical green strain, the other a new white variety.

2. Cyclopolic acid, $C_{11}H_{12}O_6$, forms colourless plates, m.p. 147–148° (decomp.). Cyclopaldic acid, $C_{11}H_{10}O_6$, forms colourless fluffy needles, m.p. 224–225°. A number of functional derivatives and degradation products of each acid has been described.

3. Cyclopolic acid is readily oxidized *in vitro* to cyclopaldic acid by heating with potassium periodate in dilute sulphuric acid solution. Both acids have been shown to be hexa-substituted benzene derivatives and to be closely related structurally to two other mould metabolic products, citrinin and mycophenolic acid.

4. Cyclopolic acid is believed to be *5-formyl-4-hydroxy-6-hydroxymethyl-2-methoxy-3-methylbenzoic acid* and cyclopaldic acid is believed to be *5:6-diformyl-4-hydroxy-2-methoxy-4-methylbenzoic acid* or its tautomer.

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APPENDIX

Antifungal Tests

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Gladiolic acid was found by Brian, Curtis & Hemming (1948) to inhibit, at low concentrations, spore germination in a number of species of fungi. Most of their assays, however, were carried out with *Botrytis allii* Munn. as test organism, using a method described earlier by Brian & Hemming (1945). In view of the close chemical relationship of gladiolic acid to dihydrogladiolic, cyclopolic and cyclopaldic acids, it was decided to compare the inhibitory effects of all four substances on the germination of conidia of *B. allii*. A culture of *B. allii* was kindly supplied by Dr P. W. Brian, of Imperial Chemical Industries Ltd., Butterwick Research Laboratories, Welwyn.

In a preliminary experiment, dilutions were made in the 'germination medium' used by Brian & Hemming (1945). Gladiolic acid showed only very slight activity at 20μg./ml., the highest concentration used. Cyclopolic acid and dihydrogladiolic acid were completely inactive at 80μg./ml., but cyclopaldic acid gave almost complete inhibition at 10μg./ml., indicating that it is appreciably more active than gladiolic acid. Brian *et al.* (1948) found that at pH 3·5 gladiolic acid gives over 95% inhibition at a concentration of 7·8μg./ml., but that activity falls off as the pH is increased. The pH of the 'germination medium' used was 5·6, which would explain the low activity of gladiolic acid in this experiment.

In a second series of tests Czapek-Dox solution, adjusted to pH 3·5, was used for making dilutions. The highest concentrations which could be tested were limited by the comparatively low solubilities of the substances. Cyclopaldic acid, in particular, has such a low solubility that the highest concentration which could be obtained was 40μg./ml., and the 160μg./ml. aqueous solution used to make the first dilution had to be kept warm in order to avoid crystallization. The results obtained are given in Table 1, the degrees of inhibition being those adopted by Brian & Hemming. 0 = No germination; (1) = less than 1% germination; 1 = 1–5% germination; 2 = 5–50% germination; 3 = 50–90% germination; 4 = 90–100%, i.e. complete germination; — = not tested.

Table 1. *Fungistatic activities of gladiolic, dihydrogladiolic, cyclopolic and cyclopaldic acids at* pH 3·5

	Concn. of acid (μg./ml.)										
Acid	320	160	80	40	20	10	5	2·5	1·25	0·625	0·312
Gladiolic	0	0	0	0	0	(1)	2	3–4	—	—	—
Dihydrogladiolic	4	4	4	4	4	—	—	—	—	—	—
Cyclopolic	3–4	4	4	4	4	—	—	—	—	—	—
Cyclopaldic	—	—	—	0	0	0	0	(1)	2	3	4

The concentration of gladiolic acid permitting less than 1% germination is 10μg./ml., which agrees well with the figure, 7·8μg./ml., given by Brian *et al.* (1948). Cyclopolic acid, at the highest concentration tested, showed a slight effect, in that a few conidia had not germinated and, in the case of those which had, the germ tubes were much shorter than in the controls. Dihydrogladiolic acid was completely inactive, the amount of growth, at the highest concentration, being equal to that in the controls. Cyclopaldic acid, as was indicated in the first experiment, is about four times as active as gladiolic acid.

SUMMARY

1. Cyclopaldic acid, at a concentration of 2·5μg./ml., permits less than 1% germination of the spores of *Botrytis allii* Munn. in Czapek-Dox medium at pH 3·5.

2. Cyclopolic acid, under the same conditions and at a concentration of 320μg./ml., shows little fungistatic activity.

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Studies in the Biochemistry of Micro-organisms

86. THE MOLECULAR CONSTITUTION OF MYCOPHENOLIC ACID, A METABOLIC PRODUCT OF *PENICILLIUM BREVI-COMPACTUM* DIERCKX. PART 3. FURTHER OBSERVATIONS ON THE STRUCTURAL FORMULA FOR MYCOPHENOLIC ACID

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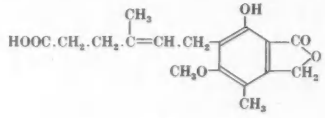
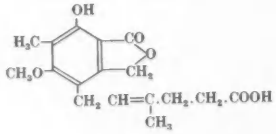
(*Received* 31 *July* 1951)

Alsberg & Black (1913) first isolated and named mycophenolic acid, $C_{17}H_{20}O_6$, from culture filtrates of *Penicillium stoloniferum* Thom. The acid was next reported by Clutterbuck, Oxford, Raistrick & Smith (1932) as a metabolic product of twelve species or strains in the *P. brevi-compactum* series, a series to which *P. stoloniferum* belongs. Clutterbuck & Raistrick (1933), as the result of an extensive chemical examination of mycophenolic acid, advanced a tentative partial structural formula for it which was modified and amplified by Birkinshaw, Bracken, Morgan & Raistrick (1948). These workers suggested structures (I) or (II) as most clearly illustrating the known properties and chemical behaviour of mycophenolic acid, and preferred (I) to (II). Further work on this subject which supports (I) is reported in the present communication.



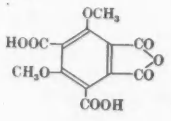
(I)



(II)

Clutterbuck & Raistrick (1933) obtained, by vigorous oxidation of mycophenolic acid monomethyl ether with alkaline potassium permanganate, a tetracarboxylic acid which was analysed as its anhydride. From this analysis the empirical formula $C_{12}H_8O_9$ was deduced for, and structure (III) was assigned to, the oxidation product.

In a recent communication on cyclopolic and cyclopaldic acids, which are metabolic products of strains of *P. cyclopium* Westling, Birkinshaw, Raistrick, Ross & Stickings (1952) showed that when methyl cyclopolate monomethyl ether is vigorously oxidized with hot alkaline potassium permanganate there is formed a tetracarboxylic acid, analysed as its anhydride which was shown conclusively to be the anhydride of 4:6-dimethoxybenzene-1:2:3:5-tetracarboxylic acid of structure (III). Hence Clutterbuck & Raistrick's anhydride, m.p. 220° (decomp.), and the anhydride of Birkinshaw *et al.* (1952), m.p. 225·5–228° (decomp.), should be one and the same substance, but proved to be different since the melting point of a mixture of Clutterbuck & Raistrick's original sample with the anhydride of Birkinshaw *et al.* was depressed to 202–206° (decomp.).



(III)

We have now repeated the permanganate oxidation of mycophenolic acid monomethyl ether substantially as described by Clutterbuck & Raistrick (1933), and have obtained their oxidation product in good yield. After exhaustive crystallization we found it to melt at 223–225° (decomp.) and not to depress the melting point, 220° (decomp.), of Clutterbuck & Raistrick's original sample, although it did depress the melting point of the anhydride of 4:6-dimethoxybenzene-1:2:3:5-tetracarboxylic acid (III), m.p. 225·5–228° (decomp.), to 203–208° (decomp.).

Since we regard as incontrovertible the evidence for the permanganate oxidation product of methyl cyclopolate monomethyl ether being assigned structure (III), it is clear that another constitution must be sought for the permanganate oxidation product of mycophenolic acid monomethyl ether.

A review of the analytical results obtained for the old sample (*A*) of the mycophenolic acid monomethyl ether oxidation product quoted from Clutterbuck & Raistrick's (1933) paper and of fresh determinations on the new sample (*B*) (see Table 1) reveals the following facts. (i) The analyses of

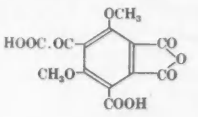
Table 1. *Analyses of oxidation products of mycophenolic acid monomethyl ether*

		C (%)	H (%)	OCH_3 (%)	Titration equivalent
(*A*)	Clutterbuck & Raistrick's original sample	48·1	2·7	20·9	80·9
		48·1	2·6	20·9	79·5
(*B*)	Present sample	48·1	2·7	19·1	81
(*C*)	Hydrogen peroxide oxidation product of (*B*)	48·5	2·7	20·7	75
	Calc. for $C_{12}H_8O_9$	48·7	2·7	20·95	74*
	Calc. for $C_{13}H_8O_{10}$	48·2	2·5	19·1	81*

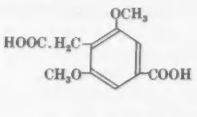
* Titrating as a tetracarboxylic acid.

samples (*A*) and (*B*) are in good agreement except for the methoxyl estimations on sample (*A*) on which much reliance was originally placed. (ii) With the exception of the methoxyl estimations on (*A*) all the other estimations are in better agreement with an empirical formula $C_{13}H_8O_{10}$ than with $C_{12}H_8O_9$ as originally assigned. This is consistent with the presence of an additional CO group and can be readily explained only on the assumption that a CO.COOH group is present as a substituent of the benzene nucleus in place of one of the carboxyl groups originally postulated. Confirmation of this hypothesis was obtained by further degradation with hydrogen peroxide at 0° of the sodium salt of the permanganate oxidation product of mycophenolic acid monomethyl ether, which then afforded in good yield the anhydride of a dimethoxybenzene tetracarboxylic acid (*C*) structure (III), $C_{12}H_8O_9$, identical with the permanganate oxidation product obtained from methyl cyclopolate monomethyl ether.

Structure (IV), i.e. the anhydride of 3:4:5-tricarboxy-2:6-dimethoxyphenylglyoxylic acid, $C_{13}H_8O_{10}$, for the permanganate oxidation product of mycophenolic acid monomethyl ether has been



(IV)



(V)

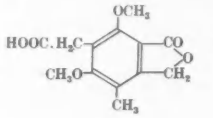
established as follows. The permanganate oxidation product was refluxed in a stream of nitrogen with hydriodic acid and red phosphorus when it was demethylated and 1·97 molecular equivalents of carbon dioxide were evolved after 4 hr. heating. The resulting decarboxylation product was isolated as a cream solid which was remethylated with dimethyl sulphate and anhydrous potassium carbonate in dry acetone giving, after alkaline hydrolysis of the methylation product, 4-carboxy-2:6-dimethoxyphenylacetic acid (structure V) as colourless needles, m.p. 286–288°, not depressing the melting point of an authentic synthetic specimen, m.p. 284–285°. This identification was confirmed by converting the decarboxylation product (V) into its dimethyl ester which was obtained as colourless jagged needles, m.p. 107·5–109° not depressed on admixture with an authentic synthetic specimen of the dimethyl ester of 4-carboxy-2:6-dimethoxyphenylacetic acid, m.p. 106·5–108°.

This experimental evidence establishes two important points. (*a*) The arguments advanced by Birkinshaw *et al.* (1948) for the constitution of mycophenolic acid (I or II) were based in part on structure (III) for its permanganate oxidation product, from which it was concluded that mycophenolic acid is a hexasubstituted benzene derivative. This conclusion has now been doubly proved with the establishment of structure (IV) for the permanganate oxidation product and structure (III) for the further degradation product obtained from it with hydrogen peroxide. (*b*) The CO.COOH side chain in (IV) must arise by oxidation of one of the substituents in the benzene ring in mycophenolic acid. It must therefore arise from the $HOOC.CH_2.CH_2.C(CH_3)=CH.CH_2$ side chain, since this is the only substituent containing more than one carbon atom. Hence it follows that this side chain must lie between the hydroxyl and methoxyl groups in mycophenolic acid. Structure (II) for mycophenolic acid must therefore now be abandoned and structure (I), which was that preferred by Birkinshaw *et al.* (1948), becomes almost certain, except for: (i) a slight doubt as to which of the two nuclear hydroxyl groups in normycophenolic acid is methylated in mycophenolic acid itself; (ii) a slight doubt as to the relative positions in (I) of the CO and CH_2 groups in the phthalide ring

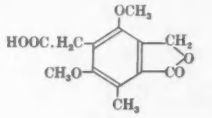
i.e. as to whether the potential CH_2OH group is in the *ortho* position to $—CH_3$ or *meta* to it.

In an attempt to resolve the doubt expressed in (ii) the aldehyde, $C_{13}H_{14}O_5$, obtained by ozonolysis of mycophenolic acid monomethyl ether (Birkinshaw *et al.* 1948), was oxidized with alkaline silver nitrate to the corresponding carboxylic acid, $C_{13}H_{14}O_6$. Since the $—CH_2.CHO$ group in the aldehyde $C_{13}H_{14}O_5$ and hence also the $—CH_2.COOH$ group in the acid $C_{13}H_{14}O_6$ arises from the long side

chain in mycophenolic acid monomethyl ether, it now follows that there are only two possible structures, i.e. (VI) or (VII), for the acid $C_{13}H_{14}O_6$, of which (VI) is preferred. It was hoped to confirm structure (VI) by decarboxylating the acid by

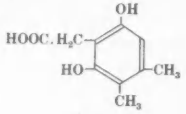


(VI)



(VII)

heating it with copper chromite and quinoline and thus obtaining 5:7-dimethoxy-4:6-dimethylphthalide, which has been isolated as a degradation product of cyclopolic acid and has been synthesized (Birkinshaw *et al.* 1952). Unfortunately, although carbon dioxide was evolved, no crystalline decarboxylation product could be isolated. However, indirect support for structure (VI) was obtained by heating the acid with hydriodic acid and red phosphorus. One molecule of carbon dioxide was evolved and a product, $C_{10}H_{10}O_3$, m.p. 184–185°, was isolated, which gives no colour with ferric chloride and is insoluble in aqueous sodium bicarbonate though readily soluble in aqueous sodium carbonate. We believe it to be one of the two possible lactones derived from 2:6-dihydroxy-3:4-dimethylphenylacetic acid (VIII).



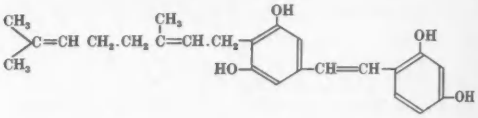
(VIII)

The loss of a molecule of carbon dioxide under these experimental conditions is much more readily explained from structure (VI) than from (VII). It is known that the $—CH_2.COOH$ side chain is stable to boiling hydriodic acid (cf. the stability of V). Hence, it is probable that the molecule of carbon dioxide which is evolved arises from the potential carboxyl group in the phthalide ring, the CH_2OH group being reduced to CH_3. On the basis of (VI) this COOH group would be situated both ortho to one and para to the other of the two hydroxyl groups formed by demethylation of the parent acid. It is our experience that a carboxyl group so placed is readily removed on heating with hydriodic acid and red phosphorus, whereas a COOH group meta to both the hydroxyl groups, such as is necessitated by (VII), would not be readily eliminated under these experimental conditions.

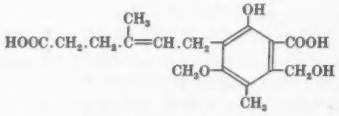
It is hoped to synthesize (VIII) and thus decide between (VI) and (VII) as the structure of the acid $C_{13}H_{14}O_6$, and so finally settle the constitution of normycophenolic acid. The only outstanding point with regard to the constitution of mycophenolic acid itself would then be the relative positions of its hydroxyl and methoxyl groups.

Support for structure (I) for mycophenolic acid is afforded by the work of Barer, Cole & Thompson (1949) who examined the infrared spectra, with the aid of a reflecting microscope, of a minute crystal of 'bialin' obtained from cultures of *P. bialowiezense* Zaleski (a synonym of *P. brevi-compactum* Dierckx) and compared it with a similar small crystal of mycophenolic acid obtained from *P. brevi-compactum* Dierckx. The two compounds were shown to be identical. In addition, however, their results suggested that the 5′-carboxy-3′-methylpent-2′-enyl grouping was *ortho* to the OH and OCH_3 groups in mycophenolic acid as required in structure (I).

The tree *Chlorophora excelsa* is the source of iroko, one of the principal commercial hardwoods of tropical Africa. Iroko, during recent years and because of the shortage of teak, has been extensively used for the construction of laboratory benches. King & Grundon (1949, 1950) have isolated from iroko, by ether extraction, a new polyphenolic body which they have named chlorophorin and which they have shown to have structure (IX). Comparison of this structure with that of mycophenolic acid (X), written with the phthalide ring opened, shows a noteworthy resemblance between the structures of these two naturally occurring compounds and illustrates the essential unity, from the point of view of comparative biochemistry, in some of the metabolic processes of microscopic fungi at one extreme of the plant kingdom and those of a forest tree at the other extreme.



(IX) Chlorophorin



(X) Mycophenolic acid (hydrated)

EXPERIMENTAL

Oxidation of mycophenolic acid monomethyl ether with hot alkaline $KMnO_4$. *Isolation of (IV)*

The method used was substantially that described by Clutterbuck & Raistrick (1933). A solution of mycophenolic acid monomethyl ether (5·0 g.) in aqueous KOH (2%; 100 ml.) was refluxed, and finely ground $KMnO_4$ (30 g.) was

added continuously in 1–2 g. portions. All the $KMnO_4$ was utilized. The precipitated MnO_2 was separated by filtration and washed thoroughly with hot water. The pale-yellow filtrate and washings were adjusted to pH 1–2 with HCl, and $CaCl_2$ (5 g.) was added. The precipitated calcium oxalate (0·06 g.) was separated by filtration and discarded. The filtrate was evaporated *in vacuo* at 50–55° to 40 ml. Conc. HCl (2 ml.) was added and the solution was extracted continuously with ether for 30 hr. Removal of the solvent gave a yellow gum (3·95 g.) which could not be obtained crystalline. It was therefore re-oxidized. It was dissolved in 2N-KOH (50 ml.) and refluxed. $KMnO_4$ (7·5 g.) was added in 0·3 g. portions until a permanent pink colour remained after heating for 20 min. The excess $KMnO_4$ was destroyed with Na_2SO_3, and oxalic acid was removed as calcium oxalate (1·40 g.) as described above. The acidified filtrate from the calcium oxalate was continuously extracted with ether for 10 hr. Removal of the solvent gave a pale-yellow gum which, on heating *in vacuo* at 70° for 1·5 hr., gradually solidified, wt. 2·05 g., m.p. 187–190° (decomp.). Repeated crystallization of this product from ethyl acetate-light petroleum (b.p. 60–80°) finally gave colourless microrods, m.p. 223–225° (decomp.), which did not depress the m.p. 220° (decomp.) of the original specimen of this oxidation product prepared by Clutterbuck & Raistrick (1933). On the other hand, on admixture with the anhydride of 4:6-dimethoxybenzene-1:2:3:5-tetracarboxylic acid (III), m.p. 225·5–228° (decomp.), obtained by $KMnO_4$ oxidation of the monomethyl ether of methyl cyclopolate (Birkinshaw *et al.* 1952), the mixture melted at 203–208° (decomp.). (Found, on sample dried to constant weight at 100° *in vacuo*: C, 48·1; H, 2·7; OCH_3, 19·1; titration equivalent to phenolphthalein in water, 81. $C_{12}H_8O_{10}$ requires C, 48·2; H, 2·5; $2OCH_3$, 19·1%; equivalent, titrating as a tetrabasic acid (di-acid mono-anhydride), 81.)

Brady's reagent (2·5 ml.) was added to a solution of the acid (10 mg.) in water (1 drop). On standing overnight a little yellow amorphous precipitate (0·5 mg.), which could not be obtained crystalline, was collected. It gave a deep-red colour, typical of a mono-2:4-dinitrophenylhydrazone, when it was dissolved in ethanolic NaOH (Neuberg's reaction).

On addition of a few drops of conc. H_2SO_4 to a suspension of the acid in benzene containing a trace of thiophen the H_2SO_4 layer rapidly became intense brown-red in colour, changing on standing overnight to an intense greenish-blue colour which became a deep pure blue on dilution with water. Control experiments with H_2SO_4 + benzene + thiophen and also with H_2SO_4 + the acid alone, gave only a moderate orange-red colour. Under the same conditions phenylglyoxylic acid gives a deep-red colour changing to reddish violet on standing (Claisen, 1879), and the 2:4-dihydroxyphenylglyoxylic acids give characteristic colours (Hunsberger & Amstutz, 1948).

Degradation of the acid $C_{13}H_8O_{10}$ *with* H_2O_2. *Isolation of (III)*

A solution of the above acid $C_{13}H_8O_{10}$ (0·25 g.) in water (2 ml.) was neutralized with N-NaOH using phenolphthalein as an external indicator. The solution was cooled to 0° and H_2O_2 (90–100 'vol.'; 0·14 ml.) was added. After standing overnight at room temperature the colourless solution was acidified with 2N-HCl (2 ml.), saturated with NaCl and extracted with ethyl acetate (4 × 10 ml.). On removal of the solvent *in vacuo* at 50° from the dried (Na_2SO_4) extract, there remained a colourless solid (0·237 g.), m.p. 225–226·5° (decomp.), which, on crystallization from ethyl acetate-light petroleum (b.p. 60–80°) gave colourless microprisms, m.p. 225·5–227° (decomp.) not depressed on admixture with the anhydride of 4:6-dimethoxybenzene-1:2:3:5-tetracarboxylic acid, m.p. 225·5–228° (decomp.), obtained by $KMnO_4$ oxidation of the monomethyl ether of methyl cyclopolate (Birkinshaw *et al.* 1952). On the other hand, on admixture with the starting acid $C_{13}H_8O_{10}$, m.p. 223–225° (decomp.) the mixture melted at 204·5–209° (decomp.). (Found, on sample dried to constant weight at 110° *in vacuo*; C, 48·5; H, 2·7; OCH_3, 20·7; titration equivalent to phenolphthalein in water, 75. $C_{12}H_8O_9$ requires C, 48·7; H, 2·7; $2OCH_3$, 21·0%; equivalent, titrating as a tetrabasic acid (di-acid mono-anhydride), 74.)

Action of HI *and red* P *on the acid* $C_{13}H_8O_{10}$. *Isolation and identification of 4-carboxy-2:6-dimethoxyphenylacetic acid (V)*

A mixture of the acid $C_{13}H_8O_{10}$ (0·307 g.), HI (*d*, 1·7; 5 ml.) and red P (0·4 g.) was refluxed in a stream of CO_2-free N_2. The effluent gases were scrubbed first through a bubbler containing red P and water and then through bubblers containing measured amounts of aqueous 0·2N-$Ba(OH)_2$ which were titrated with 0·1N-HCl at intervals. CO_2 was evolved as follows: after 0·5 hr., 1·49 mol. equiv.; 0·75 hr., 1·89 mol. equiv.; 2·5 hr., 1·95 mol. equiv.; 4 hr., 1·97 mol. equiv. The reaction was terminated after a further ½ hr. to ensure complete reduction when P was separated by filtration and washed with water. The filtrate and washings were extracted with ethyl acetate (4 × 10 ml.). The combined orange-brown extracts were washed with water, aqueous $Na_2S_2O_3$, and again with water. Removal of the solvent *in vacuo* from the dried (Na_2SO_4) extract gave a cream solid (0·113 g.) which was methylated by refluxing for 1·5 hr. with dimethyl sulphate (0·5 ml.), dry acetone (10 ml.) and anhydrous K_2CO_3 (0·25 g.). The solvent was removed and the residue was hydrolysed for 0·75 hr. at 100° with aqueous N-NaOH (10 ml.). Acidification with HCl gave a cream solid which was extracted with ethyl acetate (4 × 15 ml.). Removal of the solvent *in vacuo* gave small gummy needles (95 mg.) which, on washing with a little cold ether gave colourless needles (50 mg.), free from gum, m.p. 278–282°. This product was purified by sublimation in a high vacuum followed by two crystallizations from aqueous methanol giving 4-carboxy-2:6-dimethoxyphenylacetic acid as colourless needles, m.p. 286–288°, the dark melt rapidly resetting on cooling and remelting at 285–286·5°. On admixture with an authentic synthetic specimen, m.p. 284–285°, remelting at 282–283°, kindly supplied by Prof. F. E. King of Nottingham University, the mixture melted at 285–285·5° resetting and remelting at 283–285°. (Found: C, 55·3; H, 5·1; OCH_3, 26·1. Calc. for $C_{11}H_{12}O_6$: C, 55·0; H, 5·0; $2OCH_3$, 25·8%.)

The above acid, $C_{11}H_{12}O_6$ (24 mg.), was refluxed for 1·5 hr. with dimethyl sulphate (0·05 ml.), acetone (5 ml.) and K_2CO_3 (50 mg.). The solvent was removed and the residue was shaken with water giving a colourless product (23 mg.), m.p. 105·5–107·5°. Crystallization from aqueous methanol gave the dimethyl ester of 4-carboxy-2:6-dimethoxyphenylacetic acid as small jagged needles, m.p. 107·5–109°, resetting and remelting at 107·5–108·5°. A synthetic specimen of this ester was prepared from the authentic synthetic acid by treatment with ethereal diazomethane and crystallization of the ester from aqueous methanol giving colourless jagged

needles, m.p. 106·5–108°. A mixture of the two specimens melted at 107–108°. King & Grundon (1950) give the melting points of the free acid as 282–284° and of the ester as 108–109°. (Found: C, 58·2; H, 6·0; OCH_3, 46·3. Calc. for $C_{13}H_{16}O_6$: C, 58·2; H, 6·0; $4OCH_3$, 46·3%.)

Oxidation of the aldehyde $C_{13}H_{14}O_5$ *with* Ag_2O. *Formation of the acid* $C_{13}H_{14}O_6$ (*VI*)

The aldehyde $C_{13}H_{14}O_5$, obtained by the ozonolysis of mycophenolic acid monomethyl ether (Birkinshaw *et al.* 1948) was treated with $AgNO_3$ and alkali according to the method of Delépine & Bonnet (1909). A solution of $AgNO_3$ (605 mg.) in water (15 ml.) was added to a solution of the aldehyde $C_{13}H_{14}O_5$, m.p. 108–111·5° (294 mg.), in warm methanol (6 ml.). To the resultant slightly turbid solution aqueous 0·5N-NaOH (8·4 ml.) was added dropwise with vigorous shaking over 1 hr. Shaking was continued for a further 0·5 hr. The resulting black precipitate was separated by filtration through kieselguhr, and the filtrate was extracted with ether (2 × 20 ml.) to remove neutral material which was discarded. The extracted aqueous solution was acidified with HCl, saturated with NaCl and re-extracted with ether (3 × 25 ml.). On removal of the solvent from the dried (Na_2SO_4) ether extract the residual gum rapidly solidified to colourless needles (270 mg.), m.p. 145–148°. Crystallization from ether-light petroleum (b.p. 40–60°) gave colourless needles (183 mg.), m.p. 150–151°, resetting and remelting at the same temperature and not depressed on admixture with the acid $C_{13}H_{14}O_6$, m.p. 151–152°, obtained, in smaller yield, by oxidation of the aldehyde $C_{13}H_{14}O_5$ with alkaline iodine (Birkinshaw *et al.* 1948). The mixed melt reset and remelted at the same temperature.

Decarboxylation of the acid $C_{13}H_{14}O_6$

(*a*) *With copper chromite and quinoline.* Although CO_2 was evolved when the acid $C_{13}H_{14}O_6$ (100 mg.) was heated in N_2 with copper chromite (100 mg.) and carefully purified quinoline (1 ml.), the decarboxylation product was obtained as a dark-coloured gum which could not be obtained crystalline.

(*b*) *With* HI *and red* P. A mixture of the acid $C_{13}H_{14}O_6$ (100 mg.), HI (*d*, 1·7; 3 ml.) and red P (0·3 g.) was refluxed in a stream of CO_2-free N_2. CO_2, evolved as follows, was estimated as described on p. 633: after 2 hr., 0·18 mol. equiv.; 7 hr., 0·50 mol. equiv.; 12 hr., 0·76 mol. equiv.; 19 hr., 1·00 mol. equiv. The reaction was terminated after 19 hr. Red P was removed by filtration and the filtrate and washings were extracted with ethyl acetate (4 × 15 ml.). The combined extracts were washed with water and aqueous $Na_2S_2O_3$. Removal of the solvent *in vacuo* from the dried (Na_2SO_4) extract gave a gummy solid (50 mg.) which was sublimed at 110–130° in a high vacuum giving a colourless solid (15 mg.), m.p. 140–160°. This was crystallized from benzene and the resulting colourless needles were resublimed giving the *decarboxylation product* (6 mg.), m.p. 184–185° unchanged on recrystallization from benzene. (Found: C, 67·1; H, 5·4. $C_{10}H_{10}O_3$ requires C, 67·4; H, 5·7%.) The product is insoluble in cold saturated aqueous $NaHCO_3$, but dissolves rapidly in aqueous 2N-Na_2CO_3. Its aqueous solution gives a yellow colour with $FeCl_3$.

SUMMARY

1. Experimental evidence is recorded which establishes the constitution of the permanganate oxidation product of mycophenolic acid monomethyl ether as the anhydride of 3:4:5-tricarboxy-2:6-dimethoxyphenylglyoxylic acid.

2. The sodium salt of this acid, on further oxidation with hydrogen peroxide, gives the anhydride of 4:6-dimethoxybenzene-1:2:3:5-tetracarboxylic acid which was believed by Clutterbuck & Raistrick (1933) to be the structure of the permanganate oxidation product of mycophenolic acid monomethyl ether.

3. This evidence confirms the preferred structure advanced for mycophenolic acid by Birkinshaw *et al.* (1948).

One of us (D.J.R.) wishes to thank the New Zealand Defence Science Corps for financial support.

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Studies in the Biochemistry of Micro-organisms

87. DIHYDROGLADIOLIC ACID, A METABOLIC PRODUCT OF *PENICILLIUM GLADIOLI* MACHACEK

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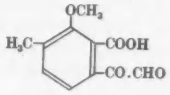
(*Received* 31 *July* 1951)

The *Penicillium gladioli* series in the subsection Fasciculata of the Asymmetrica section of species of *Penicillium* is at present represented by a single well defined species, *P. gladioli* Machacek (Raper & Thom, 1949, p. 474). Wakefield & Moore (1936), however, prefer to describe the species as *P. gladioli* McCulloch & Thom. *P. gladioli* is a common cause of the *Penicillium* rot of gladiolus corms and is occasionally responsible for serious destruction of the corms in storage.

Brian, Curtis, Grove, Hemming & McGowan (1946) showed that strains of *P. gladioli* produce a strongly antifungal and weakly antibacterial substance, gladiolic acid, $C_{11}H_{10}O_5$, the chemical properties of which 'are consistent with those that might be expected for a methoxy methyl-2-carboxyphenyl glyoxal'. Brian, Curtis & Hemming (1948) reported a detailed study of the conditions of production of gladiolic acid and of its biological properties. They showed that the acid is highly fungistatic at low pH (3·5), but that it is almost inactive at pH 7·0. Grove (1947) described a number of derivatives and breakdown products of gladiolic acid, the formation of which could best be explained by assigning to gladiolic acid the provisional structure (I).



(I)

We have now found that when a strain of *P. gladioli*, known to be descended from one of the strains used by Brian *et al.* (1948) for the production of gladiolic acid, is grown on a medium, the constituents of which are qualitatively the same as, but are present in quantitatively different amounts from, those used by Brian *et al.* (1948), there is formed in place of gladiolic acid, and in good yield, a hitherto undescribed mould metabolic product. This substance, which has the empirical formula $C_{11}H_{12}O_5$ and, as will be shown later, is readily convertible into gladiolic acid *in vitro*, we propose to name dihydrogladiolic acid. We have further found that when the same strain of *P. gladioli* is grown for the same length of time (14 days) and at the same temperature (24°) on the Raulin-Thom medium containing 7·5% glucose and with initial pH 5·0, such as was used for the production of gladiolic acid by Brian *et al.* (1948), both dihydrogladiolic acid and gladiolic acid are formed in the ratio of about 3·5 parts of the former to 1 part of the latter.

Dihydrogladiolic acid, $C_{11}H_{12}O_5$, forms colourless lustrous plates, m.p. 135–136° (decomp.), or when finely ground, m.p. 132–133° (decomp.). It has no optical activity. It contains one methoxyl group and one methyl group attached to carbon. It is a strong acid dissolving at once with effervescence in aqueous sodium bicarbonate solution and in aqueous sodium hydroxide to a colourless solution. It titrates sharply to phenolphthalein as a monobasic acid. It dissolves readily in water, ethyl acetate, ethanol and acetone, less readily in ether and is almost insoluble in benzene and in light petroleum (b.p. 60–80°). An aqueous solution of dihydrogladiolic acid gives no colour with ferric chloride but readily forms a precipitate of orange needles with Brady's reagent (0·32% 2:4-dinitrophenylhydrazine in 2N-hydrochloric acid). It gives a negative Schiff's reaction and does not reduce Fehling's solution or ammoniacal silver nitrate solution on heating for a few minutes. It gives no colour when dissolved in concentrated ammonia solution (*d*, 0·88), whereas under the same conditions gladiolic acid gives a very deep-green colour changing after some time to red and finally to orange (Brian *et al.* 1946).

The following functional derivatives have been prepared. Dihydrogladiolic acid mono-2:4-dinitrophenylhydrazone, $C_{11}H_{12}O_4{:}N.NH.C_6H_3(NO_2)_2.1H_2O$, orange microrods which do not melt up to 360°; dihydrogladiolic acid mono-semicarbazone, $C_{11}H_{12}O_4{:}N.NH.CO.NH_2.1H_2O$, colourless lustrous tablets, m.p. 130° (decomp.); dihydrogladiolic acid mono-anil, $C_{11}H_{12}O_4{:}N.C_6H_5$, colourless tetragonal plates, m.p. 161·5–162·5°, these three derivatives being soluble in cold aqueous sodium bicarbonate; crude methyl dihydrogladiolate, $C_{12}H_{14}O_5$, a thick colourless gum which could not be purified and

giving in ethanolic solution an orange-yellow precipitate with Brady's reagent. When dihydrogladiolic acid is acetylated with acetic anhydride and pyridine there is formed a diacetate, $C_{15}H_{16}O_7$, m.p. 70–70·5°, as colourless needles, which, on hydrolysis with dilute sulphuric acid, give a good yield of dihydrogladiolide, $C_{11}H_{10}O_4$, m.p. 172·5–173°, as colourless needles. Dihydrogladiolide is also formed, though in much smaller yield, by sublimation of dihydrogladiolic acid in a high vacuum. Neither dihydrogladiolide nor the above dihydrogladiolide diacetate, m.p. 70–70·5°, is soluble in cold aqueous sodium hydroxide. Further, the diacetate gives no precipitate in ethanolic solution with Brady's reagent although, under the same conditions, dihydrogladiolide forms a mono-2:4-dinitrophenylhydrazone, orange tetragonal prisms from ethyl acetate which do not melt up to 320°.

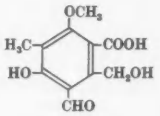
The experimental evidence presented so far justifies the working hypothesis that the following groups are present in the molecule of dihydrogladiolic acid: one OCH_3 group actually estimated; one *C*-CH_3 group actually estimated; one reactive carbonyl group, probably a CHO group explaining the formation of its mono-2:4-dinitrophenylhydrazone, mono-semicarbazone and mono-anil; one COOH group accounting for the fact that dihydrogladiolic acid is a strong acid which liberates carbon dioxide from aqueous sodium bicarbonate, and which is esterified in methyl dihydrogladiolate; one CH_2OH group, vicinal to the COOH group, to account for the formation of dihydrogladiolide with the loss of one molecule of water, dihydrogladiolide thus containing the grouping

The formation of a diacetate from dihydrogladiolic acid would then involve the acetylation of the CHO group, believed to be present in dihydrogladiolide, into $CH(O.CO.CH_3)_2$. On this hypothesis the formula for dihydrogladiolic acid may be written

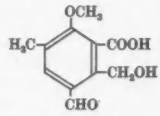
$$C_6.(H).(OCH_3).(CH_2).(CHO).(COOH).(CH_2OH) = C_{11}H_{12}O_5.$$

Dihydrogladiolic acid would then be a pentasubstituted benzene derivative, four of the substituents having C—C linkages and one of them being the OCH_3 linkage.

At this stage of the investigation of the molecular structure of dihydrogladiolic acid it became clear that there is a close similarity in the general reactions of this acid and its derivatives to those of cyclopolic acid and its corresponding derivatives. Cyclopolic acid, $C_{11}H_{12}O_6$, is a metabolic product of *P. cyclopium* Westling and a full account of the determination of its molecular structure (II) is given on p. 610 (Birkinshaw, Raistrick, Ross & Stickings, 1952*b*). The empirical formula for cyclopolic acid, $C_{11}H_{12}O_6$, differs from that of dihydrogladiolic acid, $C_{11}H_{12}O_5$, in containing one extra atom of oxygen, which may well be that contained in the phenolic group present in cyclopolic acid and accounting, on the one hand, for its intense stable purple colour with ferric chloride and, on the other hand, for the complete absence of any ferric colour with dihydrogladiolic acid. On this basis dihydrogladiolic acid might have structure (III). To test the validity of this hypothesis, which in fact proved to be correct, a number of degradation experiments was carried out.

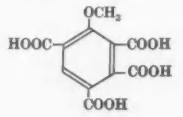


(II) Cyclopolic acid

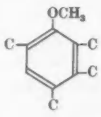


(III) Dihydrogladiolic acid

(*a*) Dihydrogladiolic acid was oxidized with hot alkaline potassium permanganate. The resulting oxidation acid, $C_{11}H_8O_9$, 4-methoxybenzene-1:2:3:5-tetracarboxylic acid of structure (IV), was obtained as colourless tetragonal prisms which on heating melted at 176–181° (decomp.), reset at about 220° and remelted at 256° (decomp.). The acid titrates sharply as a tetrabasic acid and its aqueous solution gives no colour with ferric chloride. It forms a liquid tetramethyl ester, $C_6H(OCH_3)(COOCH_3)_4$, and on demethylation gives 4-hydroxybenzene-1:2:3:5-tetracarboxylic acid, m.p. 298–299° (decomp.), an aqueous solution of which gives a stable deep orange-red colour with ferric chloride. We are greatly indebted to Mr J. F. Grove of the Imperial Chemical Industries Ltd., Butterwick Research Laboratories, Welwyn, who compared our oxidation acid with a synthetic specimen of 4-methoxybenzene-1:2:3:5-tetracarboxylic acid prepared by himself (Grove, 1952) and with his permanganate oxidation product from gladiolic acid. He found that the three specimens were identical. It follows, therefore, that dihydrogladiolic acid contains the skeleton structure (V).



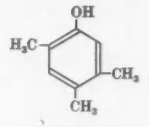
(IV)



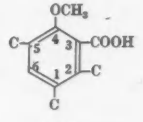
(V)

(*b*) Dihydrogladiolic acid was reduced by the Clemmensen method. The resulting reduction product, $C_{11}H_{12}O_3$, forms colourless needles, m.p. 116–116·5°, which, unlike the parent compound, are insoluble in cold aqueous sodium hydroxide—although they are soluble in the hot reagent—and

do not give in ethanolic solution any precipitate with Brady's reagent. This reduction product was heated for 5 hr. with hydriodic acid (*d*, 1·7) and a little red phosphorus. Demethylation occurred, one molecule of carbon dioxide was evolved and was estimated, and pseudocumenol, 5-hydroxy-1:2:4-trimethylbenzene of structure (VI), was formed. It was isolated as colourless needles, m.p. 71–71·5° not depressed on admixture with authentic synthetic pseudocumenol of the same melting point (for synthesis see p. 646). Its identity was confirmed by conversion into its 3:5-dinitrobenzoate, colourless needles, m.p. 179·5–180°, also not depressed on admixture with an authentic synthetic specimen. Thus, since it has been proved that dihydrogladiolic acid has the skeleton structure (V), it follows that the carboxyl group present in dihydrogladiolic acid, and evolved as carbon dioxide on heating the Clemmensen reduction product, $C_{11}H_{12}O_3$, with hydriodic acid and red phosphorus, must be in position 3 in structure (VII), since if it were in position 6, then 4-methoxybenzene-1:2:3:5-tetracarboxylic acid (IV) could not arise by the oxidation of dihydrogladiolic acid with potassium permanganate. The skeleton structure of dihydrogladiolic acid may therefore be expanded to (VII). This conclusion is confirmed by the following fact. Previous work on cyclopolic acid (Birkinshaw *et al.* 1952*b*) and on mycophenolic acid (Birkinshaw, Raistrick & Ross, 1952*a*) has shown that a carboxyl group *ortho* to a phenolic OH group or a methoxyl group is readily eliminated as carbon dioxide on heating with hydriodic acid and a little red phosphorus. A carboxyl group meta to the OH group is not, however, eliminated by this means.



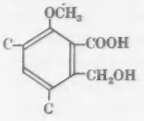
(VI)
Pseudocumenol



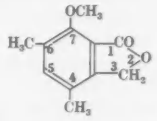
(VII)

It has already been mentioned that, in order to explain the formation of dihydrogladiolide from dihydrogladiolic acid with the loss of one molecule of water, and also to explain the properties of dihydrogladiolide, it is necessary to postulate that dihydrogladiolide is the phthalide of dihydrogladiolic acid, in which case dihydrogladiolic acid must contain the grouping

If this postulate is accepted the skeleton structure of dihydrogladiolic acid may be expanded to (VIII) and structure (IX) may be deduced for its Clemmensen reduction product since it alone explains all the known properties of this substance.



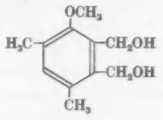
(VIII)



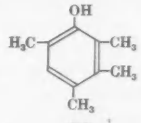
(IX)

Thus, its empirical formula is $C_{11}H_{12}O_3$, it is insoluble in cold aqueous 2N-sodium hydroxide, but dissolves on heating. It contains two methyl groups attached to carbon. It gives no precipitate with Brady's reagent. On heating with hydriodic acid and red phosphorus it would be demethylated, the phthalide ring would be slowly opened and the resulting COOH group liberated as carbon dioxide, the resulting CH_2OH group would be reduced to CH_3 and the final product would be pseudocumenol (VI). Structure (IX) for the Clemmensen reduction product, $C_{11}H_{12}O_3$, is confirmed by the fact that, although this substance gives no colour with ferric chloride, when it is demethylated but not otherwise changed the resultant product, $C_{10}H_{10}O_3$, colourless needles, m.p. 159·5–160·5°, gives a stable deep-blue colour with ferric chloride. A carboxyl group or the carbonyl group of a lactone ring ortho to a phenolic hydroxyl group would be expected to give rise to an intense colour with ferric chloride. Thus Scheussner & Voswinckel (1921) have shown that 7-hydroxy-5-methylphthalide, and Perkin & Trikojus (1926) have shown that 3-carboxy-7-hydroxy-6-methoxyphthalide, give deep-blue colours with ferric chloride. Hence, the Clemmensen reduction product $C_{11}H_{12}O_3$, is formulated as 7-methoxy-4:6-dimethylphthalide and its demethylation product, $C_{10}H_{10}O_3$, as 7-hydroxy-4:6-dimethylphthalide.

The Clemmensen reduction phthalide (IX) was further reduced with lithium aluminium hydride in ether, a process which is known to reduce lactones to diols (Adams, 1951). The resulting reduction product, a di-alcohol, $C_{11}H_{16}O_3$, was obtained in good yield as colourless needles, m.p. 97–97·5°,



(X)

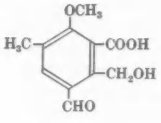


(XI)
isoDurenol

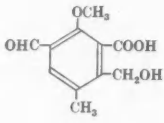
which on heating with *p*-nitrobenzoyl chloride in pyridine solution readily gave the di-*p*-nitrobenzoate, $C_{25}H_{22}O_9N_2$, as colourless needles, m.p. 157–158°. We believe the di-alcohol to be 2:3-di(hydroxymethyl)-4-methoxy-1:5-dimethylbenzene of structure (X).

The di-alcohol (X) was exhaustively reduced in glacial acetic acid solution with hydrogen and a palladium oxide catalyst. After removal of the catalyst by filtration, the reduction product still in acetic acid solution was demethylated by the addition of concentrated aqueous hydrobromic acid and boiling for 1·5 hr. The demethylated reduction product was isolated and purified and was obtained as colourless needles, $C_{10}H_{14}O$, m.p. 78–79°, not depressed on admixture with an authentic synthetic specimen of *iso*durenol, 4-hydroxy-1:2:3:5-tetramethylbenzene of structure (XI) (for synthesis see p. 647). Its identity was confirmed by conversion into its 3:5-dinitrobenzoate, colourless needles, m.p. 173·5–174·5° also not depressed on admixture with an authentic synthetic specimen. The isolation of *iso*durenol (XI) confirms the skeleton structure (V) of dihydrogladiolic acid inferred from the isolation of 4-methoxybenzene-1:2:3:5-tetracarboxylic acid (IV) by hot permanganate oxidation of dihydrogladiolic acid (see p. 636).

It is clear that one of the two CH_3 groups in the Clemmensen reduction phthalide (IX) must have arisen by reduction of the CHO group in dihydrogladiolic acid to account for the empirical formula for (IX), i.e. $C_{11}H_{12}O_3$, and its lack of carbonyl properties. Hence the structure of dihydrogladiolic acid must be either (XII) or (XIII).



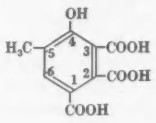
(XII)



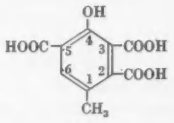
(XIII)

(*c*) A choice between (XII) and (XIII) for the structure of dihydrogladiolic acid was made as follows. Dihydrogladiolic acid was oxidized with cold alkaline potassium permanganate. The resulting oxidation product, $C_{11}H_{10}O_7$, isolated in good yield, forms colourless tetragonal microprisms, m.p. 153·5–154·5°. It contains one methyl group attached to carbon, titrates sharply as a tribasic acid, readily gives an anhydride but gives no colour with ferric chloride and no precipitate with Brady's reagent. The same tribasic acid was obtained by methylation of the oxidation acid obtained when dihydrogladiolic acid was heated at 300° in air with potassium hydroxide. This oxidation acid, $C_{10}H_8O_7$, colourless plates, m.p. 167·5–168°, which gives an intense magenta colour with ferric chloride and forms an anhydride, $C_{10}H_6O_6$, must clearly have either structure (XIV), derived from (XII), or structure (XV), derived from (XIII), the cold permanganate oxidation product of dihydrogladiolic acid being its monomethyl ether.

When the acid $C_{10}H_8O_7$ (XIV or XV) was heated with quinoline at 210–215° one molecule of carbon dioxide was evolved and the resulting decarboxylation product, $C_9H_8O_5.H_2O$, colourless tetragonal plates, m.p. 193·5–194·5° (decomp.), had the

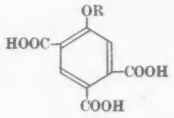


(XIV)

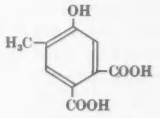


(XV)

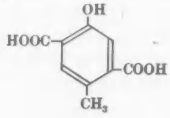
following important properties. (i) Unlike its parent substance, it gives only a pale yellow-orange colour with ferric chloride. (ii) It readily forms an anhydride, m.p. 175–175·5°. (iii) Oxidation of its methyl ether with boiling alkaline potassium permanganate gave 5-methoxybenzene-1:2:4-tricarboxylic acid (XVI, R = CH_3) identical with a synthetic specimen made by oxidizing pseudocumenol monomethyl ether (5-methoxy-1:2:4-trimethylbenzene) with boiling alkaline potassium permanganate.



(XVI)



(XVII)



(XVIII)

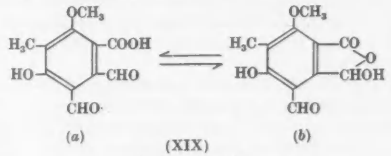
In order to obtain (XVI, R = H) from (XIV) it is necessary to remove the carboxyl group in position 3. This would lead to the decarboxylation product having structure (XVII). Similarly, to satisfy the same condition with (XV), its COOH group in position 3 must be removed, and this would lead to structure (XVIII) for its decarboxylation product. It is clear that (XVIII), being a substituted salicylic acid, would be expected to give an intense ferric colour and could not form an anhydride. On the other hand, (XVII) would not be expected to give an intense ferric colour and would readily form an anhydride. Hence, we may with confidence assign structure (XIV), i.e. 4-hydroxy-5-methylbenzene-1:2:3-tricarboxylic acid to the potassium hydroxide fusion oxidation product of dihydrogladiolic acid, and 4-methoxy-5-methylbenzene-1:2:3-tricarboxylic acid to the cold potassium permanganate oxidation product. It therefore follows that dihydrogladiolic acid itself must have

structure (XII) and is in fact 5-formyl-6-hydroxymethyl-2-methoxy-3-methylbenzoic acid.

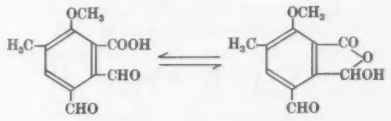
(*d*) When the neutral sodium salt of dihydrogladiolic acid is oxidized with hydrogen peroxide at 100° for 10 hr. an acid, $C_{11}H_{10}O_5$, is formed. The same acid is also obtained by treating dihydrogladiolic acid with alkaline iodine for 2 hr. at room temperature. The acid forms colourless hexagonal prisms, m.p. 216–216·5° without decomposition. *iso*-Gladiolic acid, $C_{11}H_{10}O_5$ (see p. 646), which is produced when a solution of gladiolic acid in aqueous 2 N-sodium hydroxide is boiled for 10 min., forms colourless long needles, m.p. 235·5–236·5° without decomposition. A mixture of the two acids melts at 195–205°, so that they are clearly different substances. Both acids titrate sharply in the cold as monobasic acids and, on heating, as dibasic acids (mono-acid mono-lactone) and neither of them gives a precipitate with Brady's reagent. We believe that the acid formed by oxidizing dihydrogladiolic acid either with hydrogen peroxide or with alkaline iodine is either 4-carboxy-5 (or 7)-methoxy-6-methylphthalide or 7-carboxy-6-methoxy-5-methylphthalide, but we have no experimental evidence to enable us to decide which of these formulations is correct. The structure of *iso*gladiolic acid is discussed by Grove (1952).

GLADIOLIC ACID

Workers in this laboratory (Birkinshaw *et al.* 1952*b*) found that when cyclopolic acid (II) is heated with potassium periodate in dilute sulphuric acid solution cyclopaldic acid (XIX, *a* and *b*) is formed. We



(XIX)
Cyclopaldic acid

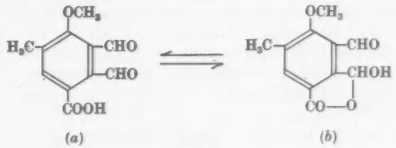


(XX)
Gladiolic acid

found that when dihydrogladiolic acid is submitted to the same reaction gladiolic acid is formed in good yield. By analogy, therefore, we arrived at structure (XX, *a* and *b*) for gladiolic acid, since this structure offers the only really feasible explanation for the transformation of dihydrogladiolic acid into gladiolic acid by the loss of two atoms of hydrogen. Our gladiolic acid was identified by direct comparison with an authentic specimen obtained from *P. gladioli* and kindly given to us by Mr J. F. Grove and also by direct comparison of specimens of *iso*-gladiolic acid obtained by heating a solution of gladiolic acid in aqueous 2 N-sodium hydroxide for 10 min. We also showed that our specimen of gladiolic acid gave, on Clemmensen reduction, the same 7-methoxy-4:6-dimethylphthalide (IX) as was obtained by Clemmensen reduction of dihydrogladiolic acid.

It is thus clear that dihydrogladiolic acid bears the same structural relationship to gladiolic acid as does cyclopolic acid to cyclopaldic acid, and that cyclopolic acid is 4-hydroxydihydrogladiolic acid and cyclopaldic acid is 4-hydroxygladiolic acid. This conclusion is supported by the fact that, as shown by Birkinshaw *et al.* (1952*b*), although cyclopolic acid and dihydrogladiolic acid have little if any antifungal activity, both cyclopaldic acid and gladiolic acid have marked antifungal activity, cyclopaldic acid being about four times as potent as gladiolic acid. The fact that cyclopaldic acid is more active than gladiolic acid is doubtless due to the presence in cyclopaldic acid of a nuclear hydroxyl group ortho to an aldehyde group and the absence of this nuclear hydroxyl group in gladiolic acid.

Our work on the structure of dihydrogladiolic and gladiolic acids had reached the above stage when one of us (H.R.) was informed by Mr J. F. Grove, in a letter dated 18 January 1951, that, having obtained a methoxybenzenetetracarboxylic acid by oxidation of gladiolic acid, he had abandoned structure (I) for gladiolic acid 2 years ago and now favoured structure (XXI, *a* and *b*). Following receipt of this information we and the Imperial Chemical Industries workers on gladiolic acid interchanged our experimental results and agreed on simultaneous publication of our findings (see Grove, 1952).



(XXI)

EXPERIMENTAL

All melting points are uncorrected. Methoxyl and equivalent determinations were carried out by one of us (D.J.R.), all other micro-analyses by Weiler and Strauss, Oxford.

History of culture

The culture of *P. gladioli* Machacek used throughout this work was obtained from the Commonwealth Mycological Institute on 5 April 1950, bearing their catalogue number C.M.I. 38567, and was given our number P 251. This strain is

descended from a culture labelled *Penicillium B* which was pathogenic on, and isolated from, Gladiolus corms by Prof. F. T. Brooks, School of Botany, University of Cambridge, and was given by him to one of us (H.R.) on 22 January 1925. It was then assigned the Ardeer catalogue number Ad. 65 and was identified by Dr C. Thom of the U.S. Department of Agriculture, who wrote 'Ad. 65 is *P. gladioli* Machacek. The same name was published independently by McCulloch and Thom but later in date'. A subculture of this strain was deposited by one of us (H.R.) in 1931 with the British National Collection of Type Cultures who gave it their catalogue number N.C.T.C. 3994, and transferred it some 3 years ago, together with the rest of their collection of fungi, to the Commonwealth Mycological Institute. The same strain (N.C.T.C. 3994) was used by Brian *et al.* (1948) in their original work on gladiolic acid, although they preferred to give it the name of *P. gladioli* McCull. and Thom. Strain C.M.I. 38567 is no longer really typical of *P. gladioli* since it is floccose rather than fasciculate and does not produce sclerotia although it freely produces conidia. On the other hand, strain Ad. 65, from which it is descended and which has been kept in laboratory culture first at Ardeer and, since 1929, in this Department, produces sclerotia freely with little formation of conidia.

Cultural conditions

The following solutions were made up. (*A*) Glucose, 2625 g. in 15 l. distilled water. This solution was sterilized separately. (*B*) Tartaric acid, 53·4 g.; ammonium tartrate, 53·4 g.; $(NH_4)_2HPO_4$, 8·0 g.; $(NH_4)_2SO_4$, 8·0 g.; K_2CO_3, 3·4 g.; $MgCO_3$, 5·4 g.; $FeSO_4.7H_2O$, 0·94 g.; $ZnSO_4.7H_2O$, 0·94 g.; distilled water, 20 l. This solution was adjusted to pH 5·0 by the addition of 2 N-NaOH, and 200 ml. quantities were distributed in each of one hundred 1 l. conical flasks, plugged with cotton wool and sterilized. After sterilization, 150 ml. of glucose solution (*A*) were added to each of the 100 flasks under aseptic conditions. Each flask was then inoculated with *P. gladioli* (C.M.I. no. 38567) grown at 24° on malt agar slopes for approximately 3 weeks, a spore suspension from each slope, prepared with sterile distilled water, being used for inoculating three flasks. The hundred flasks were then incubated in the dark at 24°.

After 4 days, growth had extended over the whole surface of the culture medium which, after 9 days, was pale yellow in colour while the upper surface of the mycelial felt was white with a few dull brown patches where the mycelial reverse was exposed to the air. After 14 days, the upper surface of the mycelium was pale cream and the lower surface dull greenish yellow in colour. The culture medium was still pale yellow.

Isolation of dihydrogladiolic acid

After 14 days' incubation in the dark at 24° the culture medium from each batch of one hundred flasks was filtered through a coarse filter paper. The pH of a representative sample of the culture filtrate was determined colorimetrically and the residual glucose was determined polarimetrically. The pH of the culture filtrate was then adjusted to pH 4·0 by the addition of 2 N-NaOH (12–15 ml./l.). Activated charcoal (British Drug Houses; 5 g./l.) was added and the suspension was stirred vigorously for 3 hr. at room temperature. After standing at 0° overnight, the bulk of the supernatant liquid was decanted from the charcoal and discarded. The thick sludge remaining was drained by filtration and the damp charcoal + adsorbate was dried *in vacuo* over conc. H_2SO_4 and then over P_2O_5 for several days. The dried charcoal was added cautiously to ether (1 l.), the sludge was placed in a percolator and the charcoal was exhaustively extracted with ether under reflux. The rate of percolation was slow and several days were required for complete extraction of ether-soluble material.

The charcoal + adsorbate from batch no. 1 was fractionally extracted with ether in an attempt to separate dihydrogladiolic acid from any other metabolic products present. In the first extraction of 12 hr. *dihydrogladiolic acid* crystallized from the ethereal extract (1500 ml.) as colourless needles (4·7 g.), m.p. 128–129·5° (decomp.), which were removed by filtration and washed with a little cold ether (20 ml.). The filtrate from this extract and the ethereal solutions from subsequent fractional extractions of the charcoal were separately evaporated. The resulting gummy crystals in each case were triturated with cold ether, filtered and washed with a little ether.

In the extraction of charcoal + adsorbate from batch no. 2 only two fractional extractions with ether were carried out. In each case dihydrogladiolic acid crystallized from the ethereal extracts as colourless needles which were removed by filtration, washed with cold ether (30 ml.) and dried. The yields obtained from batches 1 and 2 and melting points are given in Table 1, column 5.

Finally, the ether mother liquors from all fractions of batches 1 and 2 were combined. On removal of the solvent there remained a yellow gum (21·5 g. from 200 flasks) containing some needles from which, however, no other pure crystalline product could be isolated. A portion of this gum, when dissolved in conc. NH_3, gave a dark-green solution indicative of the presence of gladiolic acid.

Table 1. *Details of extraction of dihydrogladiolic acid from charcoal adsorbates of culture filtrates of* P. gladioli *C.M.I.* 38567

Batch no.	Glucose by polarimeter (% w/v)	pH	Extraction time (hr.)	Dihydrogladiolic acid (g.)	Dihydrogladiolic acid M.p. (decomp.) (°)	Total extraction time (hr.)	Total dihydrogladiolic acid (g.)
1	2·20	3·2	12	4·7	128–129·5		
				1·7	120–124		
			20	4·2	125–127		
			20	3·1	122·5–125	96	16·3
			36	2·4	122–124		
			8	0·2	122–125		
2	1·18	3·2	20	6·8	129–131	95	17·7
			75	10·9	128–130		

Isolation of dihydrogladiolic and gladiolic acids from P. gladioli *grown on Raulin-Thom medium (7·5 % glucose, initial* pH *5·0)*

Fifty 1 l. conical flasks, each containing 350 ml. of Raulin-Thom medium (7·5 % glucose, initial pH 5·0), were sterilized, inoculated with *P. gladioli* C.M.I. 38567 and incubated at 24° in the dark for 14 days. The culture filtrate from these flasks was then worked up in the same way as is described on p. 640. The following results were obtained. Residual glucose by polarimeter, 0·44 %; pH 4·5; crops of crystalline material obtained after a total extraction time of 47·5 hr., 5·95 g.; m.p. 128·5–129·5°; 4·9 g., m.p. 123–127°; 1·8 g., m.p. 120–123°; 0·4 g., m.p. 123–125°; total weight of isolated solid, 13·05 g.; residual gum, 9·7 g.

Proportionate amounts of all crops were combined. The resulting crude solid (1·64 g. equivalent to one-eighth of the total solids) was crystallized from ethyl acetate (66 ml.) + benzene (264 ml.). Pure dihydrogladiolic acid (0·74 g.), m.p. 132–133° (decomp.), was thus obtained as colourless lustrous plates. The mother liquors were evaporated to dryness at 50° *in vacuo* giving a slightly gummy solid which was triturated with cold benzene (5 ml.), filtered and washed with benzene (5 ml.). The benzene filtrate and washings were shown to contain no gladiolic acid. The colourless solid (0·50 g.), m.p. 144–146°, remaining on the filter was shown as follows to consist of crude gladiolic acid. A portion of it (100 mg. equivalent to one-fifth) was fractionally sublimed in a high vacuum. The fraction subliming between 125 and 130° (50 mg.), m.p. 156·5–158°, was collected and resublimed at the same temperature giving gladiolic acid (41 mg.), m.p. 157·5–158·5°, which on admixture with an authentic specimen (ex *P. gladioli* from I.C.I.), m.p. 159–159·5°, melted at 158–159°. The mixed melt reset and remelted at 157–158°. The sublimed sample also gave the characteristic intense colour reaction with conc. NH_3. Thus, calculating on the total solids isolated from fifty flasks, the yields obtained were as follows. (*a*) Dihydrogladiolic acid, m.p. 132–133° (decomp.), 5·9 g. equiv. to 340 mg./l. of culture solution. (*b*) Gladiolic acid, m.p. 157·5–158·5°, 1·64 g. equiv. to 94 mg./l. of culture solution.

Purification of dihydrogladiolic acid

Most of the fractions of dihydrogladiolic acid separating from ether and given in Table 1 were already of a good degree of purity and were readily completely purified by one or two crystallizations from ethyl acetate (50 ml./g.) + benzene (200 ml./g.). For example, the first fraction (4·7 g.), m.p. 128–129·5° (decomp.), from batch 1, on crystallization from these solvents, gave pure dihydrogladiolic acid as colourless lustrous plates (3·8 g.), m.p. 135–136° (decomp.), or when finely ground m.p. 132–133° (decomp.).

An attempt was made to isolate other metabolic products, including gladiolic acid, from the combined ethyl acetate-benzene mother liquors from all fractions of crude dihydrogladiolic acid from batch 1. These liquors were evaporated to 150 ml. at 45° *in vacuo*. Crude dihydrogladiolic acid (2·0 g.), m.p. 128–129° (decomp.), separated and was collected by filtration. The filtrate was evaporated at 50° *in vacuo* giving a gummy solid, trituration of which with cold benzene (10 ml.) gave further crude dihydrogladiolic acid (2·6 g.), m.p. 116–120° (decomp.), from which, or from the evaporated benzene mother liquors, no other pure compound could be isolated by fractional crystallization from water. Finally, sublimation of the crude dihydrogladiolic acid, m.p. 116–120° (decomp.) in a high vacuum at 140°, at which temperature gladiolic acid readily sublimes, gave a sublimate, m.p. 150–155° depressed to 134–138° on admixture with gladiolic acid, m.p. 159–159·5°. The sublimate probably consisted of crude dihydrogladiolide since its melting point was not depressed on admixture with pure dihydrogladiolide, m.p. 172–172·5° (see p. 642).

General properties of dihydrogladiolic acid

Dihydrogladiolic acid forms colourless lustrous plates which melt at 135–136° or, when finely ground, at 132–133°. It melts with effervescence and loss of water to a clear straw-coloured liquid which does not reset on cooling. (Found: C, 58·9, 58·9; H, 5·3, 5·1; OCH_3, 13·9; *C*-CH_3, 5·5; equiv. by titration, 223. $C_{11}H_{12}O_5$ requires C, 58·9; H, 5·4; $1OCH_3$, 13·9; $1\,C\text{-}CH_3$, 6·7 %; equiv. titrating as a monobasic acid, 224.) A 1·5 % solution in methanol shows no optical activity when viewed through a 2 dm. tube. The acid dissolves readily in water, ethyl acetate, ethanol and acetone, is slightly soluble in ether, but is almost insoluble in benzene and light petroleum (b.p. 60–80°). It dissolves at once with effervescence in cold aqueous $NaHCO_3$ and in aqueous 2 N-NaOH to a colourless solution. Its aqueous solution gives the following reactions: (*a*) no colour with $FeCl_3$; (*b*) a flocculent precipitate of orange needles with Brady's reagent (0·32 % 2:4-dinitrophenylhydrazine in aqueous 2 N-HCl); (*c*) a negative reaction with Schiff's reagent; (*d*) does not reduce Fehling's solution or ammoniacal $AgNO_3$ even on boiling for a few minutes; (*e*) with bromine water slowly gives a turbidity insoluble in excess of the reagent even on standing for several hours at room temperature. The solid acid gives no colour on solution in conc. aqueous NH_3 (*d*, 0·880) (cf. gladiolic acid), but dissolves in cold conc. H_2SO_4 to a yellow solution which becomes a deeper yellow on warming.

Functional derivatives

Dihydrogladiolic acid mono-2:4-dinitrophenylhydrazone. Brady's reagent (40 ml.) was added to a solution of dihydrogladiolic acid (63 mg.) in hot water (10 ml.). The flocculent precipitate of orange microneedles which rapidly formed was removed by filtration, washed with water and dried, wt. 119 mg. Crystallization from ethanol gave *dihydrogladiolic acid mono-2:4-dinitrophenylhydrazone* as orange microrods which on heating commenced to shrink at 198° and decomposed as the temperature was raised, but did not melt up to 360°. (Found: C, 48·2; H, 4·3; N, 13·2. $C_{17}H_{16}O_8N_4 . 1H_2O$ requires C, 48·3; H, 4·3; N, 13·3 %.) The water of crystallization could not be removed except by heating at a high temperature when obvious decomposition occurred. The compound dissolves slowly in cold aqueous $NaHCO_3$ giving a yellow solution. It gives a red Neuberg reaction (solution in cold ethanolic NaOH) confirming a mono-derivative.

Dihydrogladiolic acid mono-anil. A solution of dihydrogladiolic acid (100 mg.) in aqueous acetone (80 %; 2·5 ml.) was warmed, and aniline (0·1 ml.) was added. The colourless tetragonal prisms, formed after standing overnight at room temperature, were removed by filtration, washed with aqueous acetone and dried, wt. 93 mg., m.p. 161·5–162·5°. Crystallization from aqueous methanol gave *dihydrogladiolic acid mono-anil* as glistening colourless tetragonal plates of the same melting point. (Found: C, 67·9; H, 5·4; N, 4·2;

OCH_3, 10·3. $C_{17}H_{17}O_4N$ requires C, 68·2; H, 5·7; N, 4·7; $1OCH_3$, 10·4%.) The compound is readily soluble in methanol, but only slightly soluble in water. It dissolves slowly in cold aqueous $NaHCO_3$ and rapidly in cold 2N-NaOH.

Dihydrogladiolic acid mono-semicarbazone. A solution of dihydrogladiolic acid (45 mg.) in hot water (10 ml.) was mixed with a solution of semicarbazide hydrochloride (26 mg.) and anhydrous sodium acetate (23 mg.) in water (0·5 ml.), and heated at 100° for 5 min. After standing overnight at room temperature the clear solution deposited lustrous colourless tablets of *dihydrogladiolic acid mono-semicarbazone* which were collected, washed with water and dried, wt. 36 mg., m.p. 130° (decomp.) with shrinking and softening from 120°. Crystallization from water, in which the compound is readily soluble, effected no change of melting point. (Found: C, 48·2; H, 5·7; N, 13·8. $C_{12}H_{15}O_5N_3$. $1H_2O$ requires C, 48·2; H, 5·7; N, 14·0%.) The compound is soluble in aqueous $NaHCO_3$. An aqueous solution of this compound and a methanolic solution of the mono-anil both give precipitates of orange microneedles with Brady's reagent, though much more slowly than dihydrogladiolic acid itself under the same conditions. This is probably due to acid hydrolysis of the two compounds to their constituents.

Methyl dihydrogladiolate. Excess ethereal diazomethane was added to dihydrogladiolic acid (250 mg.). The acid dissolved slowly with gentle effervescence. After standing overnight, the solution was filtered. On removal of the solvent there remained a thick colourless gum (265 mg.) which did not solidify on standing. It could not be crystallized and did not sublime or distil on heating in a high vacuum at 150°. Crude *methyl dihydrogladiolate* is insoluble in N-NaOH. (Found, on sample dried in a high vacuum at room temperature: OCH_3, 24·5. $C_{12}H_{14}O_5$ requires $2OCH_3$, 26·1%.) An ethanolic solution of crude methyl dihydrogladiolate gives an orange-yellow precipitate with Brady's reagent.

Acetylation of dihydrogladiolic acid. Formation of dihydrogladiolide diacetate. A solution of dihydrogladiolic acid (30 mg.) in pyridine (0·1 ml.) and acetic anhydride (0·1 ml.) was held for 18 hr. at room temperature. Addition of water precipitated a colourless oil which slowly solidified, wt. 35 mg., m.p. 65·5–67·5°. Crystallization from aqueous methanol gave *dihydrogladiolide diacetate* as colourless needles, m.p. 70–70·5°. (Found: C, 58·5; H, 5·2; OCH_3, 10·2. $C_{15}H_{16}O_7$ requires C, 58·4; H, 5·2; $1OCH_3$, 10·1%.) The diacetate is insoluble in 2N-NaOH. Its ethanolic solution gives no precipitate with Brady's reagent on standing for 0·5 hr. Under the same conditions both dihydrogladiolic acid and dihydrogladiolide give orange precipitates with Brady's reagent.

Dihydrogladiolide. (*a*) *From dihydrogladiolide diacetate.* The diacetate (360 mg.) was heated under reflux for 4 hr. with 2N-H_2SO_4 (7 ml.). On cooling, the separated light-brown solid was collected, wt. 230 mg., m.p. 157–160°. Two crystallizations from aqueous methanol gave *dihydrogladiolide* as colourless needles, m.p. 172·5–173°, which reset and remelted at the same temperature. The trivial name dihydrogladiolide is given to this substance despite the fact that it is not strictly accurate nomenclature. (Found: C, 64·3; H, 5·1; OCH_3, 15·3. $C_{11}H_{10}O_4$ requires C, 64·1; H, 4·9; $1OCH_3$, 15·1%.) It is insoluble in N-NaOH. Its ethanolic solution gives an immediate gelatinous orange precipitate with Brady's reagent.

(*b*) Dihydrogladiolic acid (70 mg.) was heated in a high vacuum at 140° for 15 min. and gave a sublimate of colourless tetragonal microprisms (9 mg.), m.p. 171–171·5° not depressed on admixture with dihydrogladiolide, m.p. 172·5–173° prepared by method (*a*).

Dihydrogladiolide mono-2:4-dinitrophenylhydrazone. Brady's reagent (40 ml.) was added to a solution of dihydrogladiolide (30 mg.) in ethanol (200 ml.). The rapidly formed gelatinous orange precipitate was collected (45 mg.) and crystallized from ethyl acetate, giving *dihydrogladiolide mono-2:4-dinitrophenylhydrazone* as small orange tetragonal prisms which decomposed, but did not melt up to 320°. (Found: C, 52·7; H, 3·6; N, 14·1. $C_{17}H_{14}O_7N_4$ requires C, 52·8; H, 3·7; N, 14·5%.) The compound is slightly soluble in acetic acid, ethanol and ethyl acetate, and moderately soluble in pyridine. Its ethanolic solution gives a red colour in the Neuberg reaction confirming a mono-derivative.

Oxidation products of dihydrogladiolic acid

(*a*) *Oxidation of dihydrogladiolic acid with cold alkaline* $KMnO_4$. *Isolation of 4-methoxy-5-methylbenzene-1:2:3-tricarboxylic acid, methyl ether of* (*XIV*)

An aqueous solution of $KMnO_4$ (2% (w/v); 18 ml. equiv. to 3·8 atoms O) was added to a solution of dihydrogladiolic acid (200 mg.) in N-KOH (4 ml.). The mixture was held at room temperature overnight. The residual $KMnO_4$ was destroyed by the addition of $NaHSO_3$. MnO_2 was removed by filtration and washed with water. The filtrate and washings were acidified with HCl, saturated with NaCl and extracted with ethyl acetate (4 × 20 ml.). The water-washed extract was dried over Na_2SO_4 and evaporated *in vacuo* at 45–50° giving a pale-buff coloured solid (202 mg.), m.p. 154–155·5° (decomp.). Crystallization from ethyl acetate-benzene-light petroleum (b.p. 60–80°) gave *4-methoxy-5-methylbenzene-1:2:3-tricarboxylic acid* as colourless tetragonal microprisms, m.p. 153·5–154·5° (decomp.) (see also p. 644). (Found, on sample dried at 100° *in vacuo*: C, 51·9; H, 4·2; OCH_3, 12·0; *C*-CH_3, 7·5; equiv. by titration, 84. $C_{11}H_{10}O_7$ requires C, 52·0; H, 4·0; $1OCH_3$, 12·2; 1*C*-CH_3, 5·9%; equiv. titrating as a tribasic acid, 85.) An aqueous solution of the acid gives no colour with $FeCl_3$ and no precipitate with Brady's reagent.

The acid (30 mg.) was heated at 120–125° in a high vacuum giving a sublimate of colourless needles (22 mg.), m.p. 163–165°, which on crystallization from benzene-light petroleum (b.p. 60–80°) gave the *anhydride of 4-methoxy-5-methylbenzene-1:2:3-tricarboxylic acid* as colourless needles, m.p. 165–166·5°. (Found: C, 56·1; H, 3·7; OCH_3, 13·1; equiv. by titration, 79. $C_{11}H_8O_6$ requires C, 55·9; H, 3·4; $1OCH_3$, 13·1%; equiv. titrating as a tribasic acid (mono-acid mono-anhydride), 79.)

(*b*) *Oxidation of dihydrogladiolic acid with hot alkaline* $KMnO_4$. *Isolation of 4-methoxybenzene-1:2:3:5-tetracarboxylic acid* (*IV*)

A solution of dihydrogladiolic acid (0·45 g.) in 0·1N-NaOH (25 ml.) was heated under reflux, and powdered $KMnO_4$ (1·48 g., equiv. to 7·5 atoms O) was added gradually over 1·5 hr. The heating was continued for a further 20 min. Excess $KMnO_4$ was destroyed with $NaHSO_3$ and MnO_2 was removed by filtration. The filtrate and water washings were adjusted to pH 2 with HCl, and $CaCl_2$ (0·2 g.) was added. The small precipitate of calcium oxalate which

formed overnight was removed by filtration and discarded. The filtrate was acidified with conc. HCl (2 ml.), saturated with NaCl, and extracted with ethyl acetate (5 × 15 ml.). Removal of the solvent *in vacuo* from the dried (Na_2SO_4) extract gave a colourless solid (0·45 g.), m.p. 182–185° (decomp.), which, on crystallization from ethyl acetate-benzene-light petroleum (b.p. 60–80°), gave *4-methoxybenzene-1:2:3:5-tetracarboxylic acid* as colourless tetragonal microprisms (0·36 g.), which on heating shrink at 177°, soften at 180° and melt with slight effervescence at 182·5–184·5° (decomp.). (Found, on sample dried at 100° in a high vacuum: C, 46·7; H, 3·0; OCH_3, 11·1; equiv. by titration, 72. $C_{11}H_8O_9$ requires C, 46·5; H, 2·9; $1OCH_3$, 10·9%; equiv. titrating as a tetrabasic acid, 71.) The acid is very soluble in water, methanol and acetone, soluble in ethyl acetate and ether and almost insoluble in benzene. Its aqueous solution gives no colour with $FeCl_3$. All attempts to form a homogeneous anhydride failed.

Mr J. F. Grove of the Imperial Chemical Industries Ltd., Butterwick Research Laboratories, found the above specimen to melt in his apparatus at 176–181° (decomp.), resetting and remelting at 256° (decomp.), and to be identical with a synthetic specimen of 4-methoxybenzene-1:2:3:5-tetracarboxylic acid and with the same acid prepared by the permanganate oxidation of gladiolic acid (see Grove, 1952).

The acid (60 mg.) was methylated by treatment with an excess of ethereal diazomethane, when an immediate vigorous effervescence took place. Excess diazomethane was removed by distillation and the residual ethereal solution was washed with aqueous $NaHCO_3$ and water and dried over Na_2SO_4. Removal of the solvent gave a very pale-yellow gum (72 mg.) which could not be solidified or crystallized. It was purified by fractional distillation in a high vacuum in a 'cold-finger' apparatus. The small amount of oil distilling below 100° was rejected and the *tetramethyl ester of 4-methoxybenzene-1:2:3:5-tetracarboxylic acid* was obtained as a colourless viscous oil (50 mg.). (Found: C, 53·3; H, 5·0; OCH_3, 45·8. $C_{15}H_{16}O_9$ requires C, 52·9; H, 4·7; $5OCH_3$, 45·6%.) The ester is very soluble in ether and methanol and sparingly soluble in benzene.

The acid (80 mg.) was heated under reflux for 0·5 hr. with HI (*d*, 1·7; 4 ml.) and a little red P which was then removed by filtration from the hot solution. The filtrate, on cooling to 0°, deposited clusters of small blades (53 mg.) which were collected by filtration on a sintered-glass filter and washed with a little ice-cold conc. HCl. Crystallization of this product from acetone-benzene gave *4-hydroxybenzene-1:2:3:5-tetracarboxylic acid* as colourless tetragonal microprisms, which on heating shrink at 290°, soften at 296°, and melt with vigorous effervescence at 298–299°. (Found, on sample dried at 110° in a high vacuum: C, 44·3; H, 2·5. $C_{10}H_6O_9$ requires C, 44·4; H, 2·2%.) An aqueous solution of the acid gives a stable deep orange-red colour with $FeCl_3$. The acid proved to be identical with a synthetic specimen supplied by Mr J. F. Grove, i.e. same ferric reaction, no depression of melting point on mixing.

(c) *Oxidation of dihydrogladiolic acid with* (*i*) H_2O_2 *and* (*ii*) *alkaline* I_2. *Isolation of a carboxymethoxymethylphthalide*

(i) *With* H_2O_2. Dihydrogladiolic acid (150 mg.) was converted into its sodium salt by the addition of 0·1 N-NaOH (6·7 ml.). H_2O_2 ('100 vol.'; 0·4 ml.) was added and the solution was maintained at 100° for 10 hr. The solution was cooled, acidified with conc. HCl and held at room temperature overnight. The resulting colourless prisms were collected, wt. 85 mg., m.p. 209–212°. Sublimation at 155–175° in a high vacuum and two subsequent crystallizations from water gave *a carboxymethoxymethylphthalide* as large colourless hexagonal prisms, m.p. 216–216·5°, which reset and remelted at the same temperature. (Found: C, 59·4; H, 4·5; OCH_3, 14·0; titration equiv. cold, 217, hot, 110. $C_{11}H_{10}O_8$ requires C, 59·4; H, 4·5; $1OCH_3$, 14·0%; equiv. titrating as a monobasic acid, 222, as a dibasic acid (mono-acid mono-lactone), 111.) The acid dissolves with effervescence in aqueous $NaHCO_3$. Its aqueous solution gives no precipitate with Brady's reagent.

(ii) *With alkaline* I_2. 0·1 N-I_2 (25 ml.) and 0·1 N-NaOH (50 ml.) were added to a solution of dihydrogladiolic acid (112 mg.) in water (20 ml.). After 2 hr. at room temperature titration with aqueous $Na_2S_2O_3$ of an acidified portion (10 ml.) showed the absorption of 2·10 equiv. of $I_2 = 1·05$ atoms O. The remainder of the reaction solution (85 ml.) was acidified with 2 N-H_2SO_4 (5 ml.), and the liberated I_2 was removed by the addition of a little $NaHSO_3$. The colourless solution was saturated with NaCl and extracted with ethyl acetate (3 × 30 ml.). Removal of the solvent from the dried (Na_2SO_4) extract at 40° *in vacuo* gave slightly yellow microprisms (85 mg.), m.p. 206–208°. The crystals were purified by sublimation in a high vacuum at 150°, followed by one crystallization from water giving a carboxymethoxymethylphthalide as large colourless prisms, m.p. 216–216·5° not depressed on admixture with the H_2O_2 oxidation product (see § (i) above). The mixed melt reset and remelted at the same temperature.

The above phthalide (25 mg.) was methylated in the usual way with dimethyl sulphate (0·04 ml.), acetone (5 ml.) and K_2CO_3 (50 mg.). The methylated product (20 mg.), m.p. 132·5–134°, on crystallization from aqueous methanol gave *a carboxymethoxymethoxymethylphthalide* as thick colourless needles, m.p. 135–135·5°. (Found: C, 60·7; H, 5·0; OCH_3, 26·3. $C_{12}H_{12}O_5$ requires C, 61·0; H, 5·1; $2OCH_3$, 26·3%.) This ester is insoluble in cold aqueous 2 N-NaOH.

Degradation products of dihydrogladiolic acid

(*a*) *Clemmensen reduction of dihydrogladiolic acid. Isolation of 7-methoxy-4:6-dimethylphthalide* (*IX*). Granulated zinc (20 g.) was immersed in an aqueous solution of $HgCl_2$ (5%; 40 ml.) for 1 hr. A suspension of the washed amalgam (20 g.) was heated under reflux with conc. HCl (20 ml.), and a solution of dihydrogladiolic acid (0·65 g.) in warm water (20 ml.) was added gradually over 0·75 hr. Heating was continued for a further 0·5 hr. when the solution no longer gave a precipitate with Brady's reagent. On cooling, the slightly gummy product was mechanically separated from the amalgamated zinc, filtered, washed with water and dried, wt. 0·225 g., m.p. 109–111°. Sublimation in a high vacuum at 90–95° and subsequent crystallization from light petroleum (b.p. 60–80°), in which it is readily soluble, gave the *phthalide* as colourless needles, m.p. 116–116·5°, which reset and remelted at the same temperature. (Found: C, 68·5; H, 6·2; OCH_3, 16·3; *C*-CH_3, 11·9 equiv. 1·52 *C*-CH_3 groups. $C_{11}H_{12}O_3$ requires C, 68·7; H, 6·3; $1OCH_3$, 16·1; 2 *C*-CH_3, 15·6%.) The phthalide is insoluble in cold 2 N-NaOH, but dissolves in hot 2 N-NaOH. Its ethanolic solution gives no colour with $FeCl_3$ and no precipitate with Brady's reagent.

The above phthalide (44 mg.) was demethylated by heating under reflux for 20 min. with HI (*d*, 1·7; 3 ml.) and a little

red P. Hot water (10 ml.) was added and P was removed by filtration. On cooling, slightly brown needles (24 mg.), m.p. 155–156°, were deposited and were collected. They were sublimed in a high vacuum at 120° and subsequently crystallized from aqueous methanol giving the demethylation product, 7-*hydroxy*-4:6-*dimethylphthalide*, as colourless needles, m.p. 159·5–160·5°, which reset and remelted at the same temperature. (Found: C, 67·4; H, 5·7. $C_{10}H_{10}O_3$ requires C, 67·4; H, 5·7%.) Its ethanolic solution gives a stable, deep blue colour with ethanolic $FeCl_3$. Addition of water causes deepening, but no alteration in the shade, of the blue colour.

(*b*) *Prolonged action of* HI *and red* P *on the Clemmensen reduction phthalide* $C_{11}H_{12}O_3$. *Isolation of pseudocumenol* (5-*hydroxy*-1:2:4-*trimethylbenzene*) (*VI*). The above Clemmensen reduction phthalide, $C_{11}H_{12}O_3$ (90 mg.) was refluxed in a stream of CO_2-free N_2 with HI (*d*, 1·7; 4 ml.) and red P (0·3 g.). CO_2, estimated by absorption in 0·2 N-$Ba(OH)_2$, was evolved as follows. After 0·75 hr., 0·33 mol. equiv.; after 5·0 hr., 0·94 mol. equiv. During the reaction, oily globules collected in the lower part of the condenser system and on cooling readily solidified into needles. Long needles also separated from the cooled reaction liquid. The whole was extracted with ether (3 × 15 ml.) after dilution with water, and the ethereal extract was washed successively with water, aqueous $Na_2S_2O_3$, aqueous $NaHCO_3$ and again with water. On removal of the solvent from the ethereal extract there remained long needles, wt. 60 mg., m.p. 65·5–66·5°. This product was purified by sublimation *in vacuo* in a 'cold-finger' apparatus and two subsequent crystallizations from water giving pseudocumenol (5-hydroxy-1:2:4-trimethylbenzene) (VI) as fine colourless glistening needles, m.p. 71–71·5° not depressed on admixture with authentic synthetic pseudocumenol of the same melting point (for synthesis see p. 646). (Found, on sample dried over P_2O_5: C, 79·0; H, 9·2. Calc. for $C_9H_{12}O$: C, 79·4; H, 8·9%.) An aqueous solution of the above pseudocumenol gives with $FeCl_3$ a transient pale-blue colour fading to colourless within a few minutes with the rapid formation of a turbidity and, finally, with the formation of a colourless precipitate.

A solution of the above pseudocumenol (10 mg.) and 3:5-dinitrobenzoyl chloride (17·5 mg.) in pyridine (0·25 ml.) was refluxed for 0·5 hr. After cooling, N-H_2SO_4 (3 ml.) was added, and the resulting pale-yellow needles were separated by filtration, washed with water and triturated with 0·5 N-NaOH (3 ml.). Subsequent filtration and washing with water gave needles, wt. 14 mg., m.p. 176·5–177·5°, which, on sublimation in a high vacuum at 140–145°, gave *pseudocumenol* 3:5-*dinitrobenzoate* as colourless needles, m.p. 179·5–180° not depressed on admixture with an authentic synthetic specimen of the same melting point (for synthesis see p. 646). The mixed melt reset and remelted at the same temperature. (Found: C, 58·3; H, 4·4; N, 8·3. $C_{16}H_{14}O_6N_2$ requires C, 58·2; H, 4·3; N, 8·5%.)

(*c*) *Further reduction of the Clemmensen reduction phthalide* $C_{11}H_{12}O_3$. *Isolation of* 2:3-*di*(*hydroxymethyl*)-4-*methoxy*-1:5-*dimethylbenzene* (*X*). Powdered lithium aluminium hydride (0·35 g.) was refluxed for 2 hr. with ether (30 ml.), dried over a Na-K liquid alloy. Refluxing was continued while a solution of the Clemmensen reduction phthalide (0·245 g.) in dry ether (25 ml.) was added over 20 min. with constant stirring. The suspension was refluxed for a further 20 min. and was then cooled to 0°. The stirring was continued and water (5 ml.), followed by 2 N-H_2SO_4 (25 ml.) was cautiously added. The ethereal layer was separated and the aqueous layer was re-extracted with ether (3 × 25 ml.). Removal of the solvent from the combined dried (Na_2SO_4) extracts gave long colourless needles (0·23 g.), m.p. 88–88·5°, resetting and remelting at 95·5–96°. Crystallization from light petroleum (b.p. 60–80°), in which the compound is readily soluble, gave 2:3-*di*(*hydroxymethyl*)-4-*methoxy*-1:5-*dimethylbenzene* as fine glistening colourless needles, m.p. 97–97·5°, which rapidly reset and remelted at 96·5–97°. (Found: C, 67·0; H, 8·3; OCH_3, 16·0. $C_{11}H_{16}O_3$ requires C, 67·3; H, 8·2; 1OCH_3, 15·8%.)

The *di*-p-*nitrobenzoate* of the above substance was prepared by heating a solution of it (10 mg.) in pyridine (0·15 ml.) with *p*-nitrobenzoyl chloride (20 mg.) at 100° for 0·5 hr. The product was recovered in the usual way and on crystallization from methanol gave the di-*p*-nitrobenzoate as small colourless needles, m.p. 157–158°. (Found: C, 60·4; H, 4·4; N, 5·5. $C_{25}H_{22}O_9N_2$ requires C, 60·7; H, 4·5; N, 5·7%.)

(*d*) *Catalytic reduction of the di-alcohol* (*X*). *Isolation of* iso*durenol* (*XI*) (4-*hydroxy*-1:2:3:5-*tetramethylbenzene*). A solution of the above 2:3-di(hydroxymethyl)-4-methoxy-1:5-dimethylbenzene (X) (0·3 g.) in glacial acetic acid (20 ml.) was shaken with H_2 in the presence of palladium oxide catalyst (0·2 g.) supplied by Johnson, Matthey & Co. Ltd. Two further additions, each of 0·1 g., of the catalyst were made during the reduction before the uptake of H_2 finally ceased after 3·25 hr. 65 ml. H_2 (corrected for catalyst absorption and at N.T.P.) were absorbed; theoretical for absorption of 2 mol. H_2, 69 ml. The catalyst was removed by filtration and washed with a little glacial acetic acid. HBr (constant b.p.; 10 ml.) was added to the acetic acid filtrate and washings and the whole was refluxed for 1·5 hr. to demethylate the reduction product. The clear reddish reaction liquors were then adjusted to about pH 6 by the addition of aqueous 10 N-NaOH and the oily solid which thus separated was extracted with ether (2 × 40 ml.). The ether extract was washed with water and dried (Na_2SO_4). Removal of the solvent gave a gum which rapidly solidified to long slightly brown needles (0·21 g.), m.p. 72–74·5°, smelling faintly of acetic acid. This crude product was distilled in steam and gave colourless solidified globules (0·14 g.), m.p. 72–74·5°, which were collected and crystallized twice from aqueous methanol. *iso*Durenol (0·06 g.) was thus obtained as long glistening colourless needles, m.p. 78–79°, resetting on cooling and remelting at the same temperature. Admixture with authentic synthetic *iso*durenol (see p. 647), m.p. 77·5–78·5°, gave no depression of melting point, the mixture melting at 78–78·5°, resetting and remelting at 77·5–78·5°. (Found, on sample dried over P_2O_5: C, 79·7; H, 9·3. Calc. for $C_{10}H_{14}O$: C, 80·0; H, 9·4%.)

The reduction *iso*durenol (20 mg.) was heated at 110–120° with 3:5-dinitrobenzoyl chloride (31 mg.) in pyridine (0·3 ml.). The product was recovered in the usual way and crystallized from methanol giving iso*durenol* 3:5-*dinitrobenzoate* as colourless needles (18 mg.), m.p. 173·5–174·5°, which reset and remelted at 171–173°. Admixture with an authentic synthetic specimen, m.p. 173·5–174·5° (see p. 647) gave no depression of melting point, the mixed melt resetting and remelting at 171–172°. (Found: C, 59·4; H, 4·6; N, 8·2. $C_{17}H_{16}O_6N_2$ requires C, 59·3; H, 4·7; N, 8·1%.)

Potash fusion of dihydrogladiolic acid

Isolation and identification of 4-*hydroxy*-5-*methylbenzene*-1:2:3-*tricarboxylic acid* (*XIV*). A mixture of dihydrogladiolic acid (1·0 g.), KOH (5·0 g.) and water (1 ml.) was heated

in air at 160° in a Wood's metal bath. The melt was raised during 15 min. to 300° and maintained at this temperature for a further 10 min. The cooled, light brown, melt was dissolved in water (25 ml.), filtered, and the filtrate was acidified with conc. HCl. The resulting cream solid precipitate (0·86 g.) was collected. It consisted essentially of a crude potassium salt which is only slightly soluble in water, but dissolves immediately in 2N-Na_2CO_3. It was dissolved in hot water (50 ml.), adjusted to pH 5 with 2N-NaOH, acidified with excess HCl and extracted with ethyl acetate (4 × 25 ml.). Removal of the solvent from the dried (Na_2SO_4) extract by evaporation *in vacuo* at 50° gave a light-brown solid (0·41 g.). This substance, on crystallization from ethyl acetate-benzene, gave *4-hydroxy-5-methylbenzene*-1:2:3-*tricarboxylic acid* as colourless lustrous plates, m.p. 167·5–168° (decomp.). (Found, on sample dried at 100° in a high vacuum: C, 50·2; H, 3·8. $C_{10}H_8O_7$ requires C, 50·0; H, 3·4%.) The acid is very soluble in water, methanol and ethyl acetate, and is almost insoluble in benzene. It dissolves immediately with effervescence in aqueous $NaHCO_3$. Its aqueous solution gives no precipitate with Brady's reagent, but gives a stable intense magenta colour with $FeCl_3$. On methylation it gives 4-methoxy-5-methylbenzene-1:2:3-tricarboxylic acid identical with the acid, m.p. 153·5–154·5° (decomp.), obtained by cold $KMnO_4$ oxidation of dihydrogladiolic acid (see p. 642).

The KOH fusion acid (30 mg.) was heated at 155° in a high vacuum giving a sublimate of small tetragonal plates (17 mg.), m.p. 146·5–148°, which on crystallization from benzene, gave the *anhydride of 4-hydroxy-5-methylbenzene*-1:2:3-*tricarboxylic acid* as small colourless hexagonal prisms, m.p. 146·5–147°, which reset and remelted at 145–146·5°. (Found: C, 54·2; H, 2·6. $C_{10}H_6O_6$ requires C, 54·1; H, 2·7%.) The anhydride dissolves slowly in cold water. It dissolves rapidly with effervescence in aqueous $NaHCO_3$ to a deep-yellow solution which fades to a colourless solution within a few minutes. Its ethanolic solution gives a deep-magenta colour with ethanolic $FeCl_3$, the colour becoming more intense on the addition of water.

In a quantitative experiment, the KOH fusion acid, 4-hydroxy-5-methylbenzene-1:2:3-tricarboxylic acid, (205 mg.) was heated with quinoline (0·6 ml.) at 210–215° in a stream of CO_2-free N_2. CO_2, absorbed in standard baryta, was evolved as follows. After 5 min., 0·42 mol. equiv.; 20 min., 0·84 mol. equiv.; 30 min., 0·92 mol. equiv.; 45 min., 0·96 mol. equiv. In a preparative experiment, the acid (644 mg.) was heated with quinoline (3 ml.) at 210° for 25 min. 2N-HCl (30 ml.) was added, the solution was saturated with NaCl and extracted with ethyl acetate (4 × 25 ml.). Removal of the solvent from the washed and dried (Na_2SO_4) extract gave yellow gummy needles (440 mg.) which were purified by two sublimations at 125–150° in a high vacuum, followed by crystallization from ethyl acetate-benzene-light petroleum (b.p. 60–80°) giving *4-hydroxy-5-methylphthalic anhydride* as fine colourless needles (190 mg.), m.p. 175–175·5°, which reset and remelted at practically the same temperature. (Found: C, 60·4; H, 3·5. $C_9H_6O_4$ requires C, 60·7; H, 3·4%.) The anhydride is of low solubility in cold water, but dissolves readily on warming. It is readily soluble in ethyl acetate and methanol and almost insoluble in benzene and light petroleum (b.p. 60–80°). It dissolves readily in saturated aqueous $NaHCO_3$ to a yellow solution which quickly fades to colourless. Its aqueous solution gives a pale yellow-orange colour with $FeCl_3$.

The above anhydride (25 mg.) was boiled for 2 min. with water (0·5 ml.). The colourless solution was evaporated to dryness *in vacuo* over P_2O_5 and the residue was crystallized from ethyl acetate-benzene giving *4-hydroxy-5-methylphthalic acid* (XVII) as colourless lustrous tetragonal plates (19 mg.), m.p. 193·5–194·5° (decomp.). The compound softened slightly at 122°, partially melted at 142°, reset by 150° and then melted sharply at 193·5–194·5° with effervescence. When the acid was dried at 100° *in vacuo* no initial softening occurred. (Found, on sample dried at 100° in a high vacuum: C, 50·7; H, 4·9. $C_9H_8O_5$, $1H_2O$ requires C, 50·5; H, 4·7%.) Its aqueous solution gives a pale yellow-orange colour with $FeCl_3$.

A mixture of 4-hydroxy-5-methylphthalic anhydride (75 mg.), dimethyl sulphate (0·4 ml.), acetone (10 ml.) and K_2CO_3 (100 mg.) was refluxed for 1·5 hr. The solvent was removed and the residue was saponified with methanolic N-NaOH (10 ml.) for 0·5 hr. Methanol was removed, the residue was dissolved in water (10 ml.) and acidified with HCl. The resulting pale-yellow needles were collected (69 mg.) and crystallized twice from water giving 4-methoxy-5-methylphthalic acid as pale-yellow needles, m.p. 180–180·5° (decomp.). (Found: C, 57·2; H, 4·7; OCH_3, 14·8. Calc. for $C_{10}H_{10}O_5$: C, 57·1; H, 4·8; $1OCH_3$, 14·8%.)

The above acid (30 mg.) was refluxed for 0·5 hr. at 140° with acetic anhydride (0·9 ml.). The solvent was removed *in vacuo* and the residue was sublimed at 90–95° in a high vacuum giving *4-methoxy-5-methylphthalic anhydride* as colourless plates (23 mg.), m.p. 158·5–159° unchanged on crystallization from light petroleum (b.p. 60–80°), and resetting and remelting at the same temperature. (Found: C, 62·4; H, 4·4; OCH_3, 16·3. $C_{10}H_8O_4$ requires C, 62·5; H, 4·2; $1OCH_3$, 16·2%.)

A boiling solution of 4-methoxy-5-methylphthalic acid (83 mg.) in 0·1N-NaOH (10 ml.) was oxidized with $KMnO_4$ (0·14 g., equiv. to 3·4 atoms O) added gradually over 1·25 hr. The oxidation product was isolated as described on p. 642 and was recovered as a colourless solid (66 mg.), m.p. 210–212·5° (decomp.). It was crystallized from acetone-benzene giving 5-methoxybenzene-1:2:4-tricarboxylic acid (XVI, R = CH_3) as colourless tetragonal microprisms, m.p. 214–216·5° (decomp.) with shrinking from 204° and softening at 210°. The melting point was undepressed on admixture with an authentic synthetic specimen of the same melting point (for synthesis see p. 646). (Found: C, 50·3; H, 3·5; OCH_3, 12·9. Calc. for $C_{10}H_8O_7$: C, 50·0; H, 3·4; $1OCH_3$, 12·9%.) The acid is readily soluble in water, methanol and acetone and is almost insoluble in benzene.

Meldrum & Kapadia (1932) synthesized an acid which they claimed to be 4-hydroxy-5-methylphthalic acid, m.p. 244–245°. Their evidence for its structure is ambiguous. No ferric reaction was described and the methyl ether and anhydride were not prepared. Charlesworth, Rennie, Sinder & Yan (1945), who accepted Meldrum & Kapadia's orientation, synthesized what they claimed to be 4-methoxy-5-methylphthalic acid and described it as colourless crystals m.p. 166·5–167° (decomp.). No derivatives of this acid were reported.

Gladiolic acid

Action of potassium periodate on dihydrogladiolic acid. Isolation of gladiolic acid

In a quantitative experiment a solution of dihydrogladiolic acid (200 mg.) in water (15 ml.), aqueous KIO_4 (approx. 0·12N; 50 ml.) and 2N-H_2SO_4 (5 ml.) was held at room temperature for 24 hr. KI was then added to a

measured portion and the liberated I_2 was titrated with 0·1 N-$Na_2S_2O_3$. No KIO_4 had, however, been utilized and dihydrogladiolic acid (190 mg.) was recovered after extraction with ethyl acetate. The recovered acid gave no colour with conc. aqueous NH_3 indicating the absence of gladiolic acid.

A vigorous reaction occurred, however, on boiling. A solution of pure dihydrogladiolic acid (0·5 g.), KIO_4 (0·525 g.) and N-H_2SO_4 (25 ml.) was boiled for 15 min. The solution turned brown and crystals of I_2 were evolved. On cooling, fine colourless needles (0·30 g.), m.p. 140–143°, separated and were collected. Crystallization from water (10 ml.) gave gladiolic acid as fine colourless needles (0·24 g.), m.p. 157–157·5° raised to 159–159·5° by sublimation in a high vacuum at 130° and not depressed on admixture with authentic gladiolic acid of the same melting point. The mixed melt reset and remelted at 157·5–158°. (Found: C, 59·5; H, 4·8; OCH_3, 14·2; equiv. by titration, 221. Calc. for $C_{11}H_{10}O_5$: C, 59·4; H, 4·5; $1OCH_3$ 14·0%; equiv. titrating as a monobasic acid, 222.) We are indebted to Mr J. F. Grove for the authentic specimen of gladiolic acid from *P. gladioli* and also for a specimen of *iso*gladiolic acid (see next section). The specimen of gladiolic acid prepared from dihydrogladiolic acid gave the following reactions, previously recorded by the Imperial Chemical Industries Ltd. workers as typical of gladiolic acid. An aqueous solution of the acid gives a gelatinous orange precipitate with Brady's reagent and reduces ammoniacal $AgNO_3$ on heating. When the acid is dissolved in conc. aqueous NH_3 (*d*, 0·88) a green-brown colour is initially formed and rapidly darkens to a very deep brown-green colour. After 1 hr. the colour changes to deep brown, gradually changing to orange-red over 18 hr. and to orange over 3 days. Under the same conditions cyclopaldic acid gives initially a brown colour rapidly changing to deep purple and slowly fading to orange with a yellow fluorescence (Birkinshaw *et al.* 1952*b*).

Action of alkali on gladiolic acid. Isolation of iso*gladiolic acid*

A solution of gladiolic acid (44 mg., from dihydrogladiolic acid) in aqueous 2 N-NaOH (10 ml.) was refluxed for 10 min. when a test portion of the solution no longer gave a precipitate with excess of Brady's reagent. The solution was then acidified with HCl giving rapid separation of colourless needles which were collected (wt. 38 mg.), m.p. 235·5–236·5°. Crystallization from hot water (25 ml.), in which the compound is sparingly soluble, gave *iso*gladiolic acid as fine long colourless needles (32 mg.) of the same melting point. Admixture with authentic *iso*gladiolic acid, m.p. 234–235°, gave no depression of melting point, the mixed melt resetting and remelting at 232·5–234°. (Found: C, 59·5; H, 4·4; OCH_3, 14·1; equiv. by titration in ethanol, cold 219, hot 114. Calc. for $C_{11}H_{10}O_5$: C, 59·4; H, 4·5; $1OCH_3$, 14·0%; equiv. titrating as a monobasic acid, 222, as a dibasic acid, i.e. mono-acid mono-lactone, 111.) Unlike gladiolic acid, *iso*gladiolic acid dissolves in conc. aqueous NH_3 to a colourless solution and its aqueous solution gives no precipitate with Brady's reagent.

Clemmensen reduction of gladiolic acid. Isolation of 7-methoxy-4:6-dimethylphthalide (IX)

A solution of gladiolic acid (200 mg., from dihydrogladiolic acid) in hot water (12 ml.) was added during 0·5 hr. to mercury-amalgamated zinc (10 g.) heated under reflux with conc. hydrochloric acid (10 ml.). The heating was continued for a further 0·5 hr. when a test portion no longer gave a precipitate with Brady's reagent. The aqueous liquors were cooled to 30° and decanted from the amalgamated zinc and from some oily material which had separated. The decanted liquid was cooled to 0° and the colourless solid which separated was collected (wt. 45 mg.), m.p. 106–109°. Sublimation at 75–80° in a high vacuum gave 7-methoxy-4:6-dimethylphthalide as thick colourless needles (35 mg.), m.p. 115·5–116° not depressed on admixture with the phthalide, m.p. 116–116·5°, obtained by Clemmensen reduction of dihydrogladiolic acid (see p. 643).

Syntheses

(*a*) *Pseudocumenol* (*VI*). Pseudocumenol was synthesized from pseudocumenesulphonic acid (1:2:4-trimethylbenzene-5-sulphonic acid) according to the method of Reuter (1878), who employed the method of Jacobsen (1878) for the preparation of xylenols from xylenesulphonic acids. A mixture of pseudocumene sulphonic acid (1:2:4-trimethylbenzene-5-sulphonic acid) (15 g.), m.p. 112°, supplied by British Chemicals and Biologicals Ltd., Genatosan Division (Fine Chemicals), KOH (32 g.) and water (5 ml.) was heated with constant stirring in a nickel crucible over a naked flame. The contents rapidly set to a lumpy mass. At approximately 300°, the mass melted to a brown liquid and the melt was maintained at this temperature for 10 min. After cooling, water (500 ml.) and conc. HCl (75 ml.) were added, and the whole was extracted with ether (2 × 150 ml.). The brown ethereal extract was washed successively with water, aqueous $NaHCO_3$ and water. On removal of the solvent from the dried (Na_2SO_4) ethereal extract there remained long needles (3·0 g.) which were purified first by distillation in steam giving colourless needles (2·1 g.), m.p. 62·5–65°, followed by two crystallizations from water. Pseudocumenol was thus obtained as long glistening colourless needles, m.p. 71–71·5°, which gave the same $FeCl_3$ reaction as did the pseudocumenol obtained by degradation of the Clemmensen reduction phthalide (see p. 644). (Found, on sample dried over P_2O_5: C, 79·5; H, 9·1. Calc. for $C_9H_{12}O$: C, 79·4; H, 8·9%.) There are three possible monohydroxy derivatives of pseudocumene (1:2:4-trimethylbenzene). (*a*) Pseudocumenol (5-hydroxypseudocumene), the melting point of which, given by a number of authors, ranges from 70 to 72°; (*b*) 6-hydroxypseudocumene, m.p. 95° (Jacobsen, 1886); (*c*) 3-hydroxypseudocumene, m.p. 62° (Morgan & Pettet, 1934).

A portion of the above synthetic pseudocumenol was converted into its 3:5-dinitrobenzoate by the same method as is described on p. 644. Crystallization from methanol gave *pseudocumenol 3:5-dinitrobenzoate* as colourless needles, m.p. 179·5–180°, which reset and remelted at the same temperature. (Found: C, 58·3; H, 4·3; N, 8·2. $C_{16}H_{14}O_6N_2$ requires C, 58·2; H, 4·3; N, 8·5%.)

(*b*) *5-Methoxybenzene-1:2:4-tricarboxylic acid* (*XVI*, R = CH_3). A mixture of pseudocumenol (0·5 g.), dimethyl sulphate (1 ml.), acetone (15 ml.) and K_2CO_3 (0·6 g.) was refluxed for 1·5 hr. The solvent was removed and the residue was shaken vigorously for 15 min. with aqueous 0·5 N-KOH (30 ml.). The insoluble gum was extracted with ether (2 × 25 ml.). Removal of the solvent from the dried (Na_2SO_4) ethereal extracts gave crude pseudocumenol methyl ether as a colourless gum (0·44 g.) which was not further purified. A suspension of this gum in aqueous

0·1 N-NaOH (50 ml.) was refluxed, and powdered $KMnO_4$ (3·8 g.) was added in portions over 6 hr. Excess $KMnO_4$ was destroyed with $NaHSO_3$, and MnO_2 was removed by filtration and washed with water. The filtrate and washings were adjusted to pH 2 with HCl, and $CaCl_2$ (0·5 g.) was added. The resulting precipitate of calcium oxalate (0·19 g.) was filtered after 18 hr. and discarded. The filtrate was acidified with conc. HCl (5 ml.), saturated with NaCl and extracted with ethyl acetate (4 × 30 ml.). Removal of the solvent from the extract *in vacuo* at 45° gave an almost colourless solid (0·17 g.) which was crystallized twice from acetone-benzene-light petroleum (b.p. 60–80°) giving 5-methoxybenzene-1:2:4-tricarboxylic acid as colourless tetragonal microprisms, m.p. 214–216·5° (decomp.). (Found: C, 49·8; H, 3·4; OCH_3, 12·7; equiv. by titration, 81. Calc. for $C_{10}H_8O_7$: C, 50·0; H, 3·4; $1OCH_3$, 12·9%; equiv. titrating as a tribasic acid, 80.) Buehler, Spees & Sanguinetti (1949) give the melting point of 5-methoxybenzene-1:2:4-tricarboxylic acid as 224–225° with effervescence.

(*c*) iso*Durenol*, 4-*hydroxy*-1:2:3:5-*tetramethylbenzene* (*XI*). *iso*Durenesulphonic acid (1:2:3:5-tetramethylbenzene-4-sulphonic acid) was prepared essentially as described by Smith & Cass (1932). *iso*Durene (15 g.), supplied by British Chemicals and Biologicals Ltd., Genatosan Division (Fine Chemicals), was shaken for 10 min. at room temperature with conc. H_2SO_4 (30 ml.). The brownish solution was then poured, with stirring, into crushed ice (30 g.) surrounded by an ice bath. The precipitated solid was removed by filtration on a sintered-glass filter, pressed and dried on a porous plate, wt. 28 g., m.p. 72–76°. A solution of the crude product (14 g.) in water (35 ml.), cooled in ice, was saturated with gaseous HCl. The precipitated solid was collected and air-dried giving *iso*durenesulphonic acid (11 g.) as a nearly colourless solid, m.p. 75–79°. (Smith & Cass give m.p. 79° for pure *iso*durenesulphonic acid.)

*iso*Durenol was prepared by fusion with KOH of the above *iso*durenesulphonic acid by the same method as was used for the preparation of pseudocumenol (see syntheses, § *a*). The crude *iso*durenol obtained by steam distillation was purified by repeated crystallization from aqueous methanol giving pure *iso*durenol as long glistening colourless needles, m.p. 77·5–78·5°, which reset on cooling and remelted at the same temperature. (The melting point of *iso*durenol given by a number of authors ranges from 79 to 81°.) (Found, on sample dried over P_2O_5: C, 79·9; H, 9·5. Calc. for $C_{10}H_{14}O$: C, 80·0; H, 9·4%.)

A portion of the above *iso*durenol (20 mg.) was heated at 110–120° for 0·5 hr. with 3:5-dinitrobenzoyl chloride (30 mg.) in pyridine (0·3 ml.). The product was recovered in the usual way and was crystallized from methanol giving *isodurenol* 3:5-*dinitrobenzoate* as colourless needles (26 mg.), m.p. 173·5–174·5°, resetting and remelting at 171·5–172°. (Found: C, 59·1; H, 4·7; N, 8·2. $C_{17}H_{16}O_6N_2$ requires C, 59·3; H, 4·7; N, 8·1%.)

SUMMARY

1. A hitherto undescribed mould metabolic product, dihydrogladiolic acid, has been isolated from culture filtrates of *Penicillium gladioli* Machacek grown on a modified Raulin-Thom solution.

2. Dihydrogladiolic acid, $C_{11}H_{12}O_5$, forms colourless lustrous plates, m.p. 135–136° (decomp.). It is believed to be 5-formyl-6-hydroxymethyl-2-methoxy-3-methylbenzoic acid. A number of its functional derivatives and degradation products have been described.

3. Dihydrogladiolic acid is readily oxidized *in vitro* to gladiolic acid by heating with potassium periodate in dilute sulphuric acid solution. Gladiolic acid is believed to be 5:6-diformyl-2-methoxy-3-methylbenzoic acid, or its tautomer.

4. Cyclopolic and cyclopaldic acids from *P. cyclopium* Westling (Birkinshaw *et al.* 1952*b*) are thus 4-hydroxydihydrogladiolic acid and 4-hydroxy gladiolic acid respectively.

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Gladiolic Acid, a Metabolic Product of *Penicillium gladioli*

1. STRUCTURE

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Wilkins & Harris (1943, 1944) found that *Penicillium gladioli* Machacek (≡*P. gladioli* McCull. & Thom) produced an antibacterial substance when grown on liquid nutrient media, and Brian *et al.* (1946) showed that culture filtrates of *P. gladioli* were toxic to fungi, the fungistatic effect being more pronounced than the antibacterial effect. The production and isolation of the active material named gladiolic acid, $C_{11}H_{10}O_5$, m.p. 160°, have already been described (Brian *et al.* 1946) (Brian, Curtis & Hemming, 1948), and chemical work designed to elucidate the structural formula of this compound has now been completed. Early in the investigation it was realized that in solution under the influence of chemical reagents, gladiolic acid and certain of its derivatives, showed keto-lactol tautomerism (Grove & Willis, 1951) and could not be represented by a single structural formula of the conventional type. The present paper is concerned mainly with the chemistry of gladiolic acid and with the chemical evidence in support of the tautomeric structural formulae; the determination, by spectrographic methods, of the precise structures of gladiolic acid and some of its derivatives in the solid state, will be the subject of a separate communication.

Three compounds without antifungal properties have also been isolated, though in very low yield, from the crude solid obtained by charcoal extraction of the culture filtrates of *P. gladioli* and elution of the charcoal with ether, and their respective structural relationships to gladiolic acid have been determined.

The crude gladiolic acid, m.p. 140–155°, obtained by the charcoal extraction procedure is purified by solution in ether and extraction into sodium bicarbonate. Acidification of the bicarbonate extract and repeated crystallization of the product from water yields pure gladiolic acid, m.p. 160°. The bicarbonate-insoluble fraction normally consists of a phenolic substance, $C_{10}H_{10}O_3$, m.p. 158°, giving a deep-blue colour with ferric chloride, and is present to the extent of about 0·3% in the crude gladiolic acid. As is described below, Clemmensen reduction of gladiolic acid followed by demethylation of the reduction product gives a phenolic substance, $C_{10}H_{10}O_3$, identical with the above compound. One particular batch of crude gladiolic acid was found to have an abnormally low melting point (120–140°) and a much reduced solubility in ether. Crystallization of the ether-insoluble fraction and fractional crystallization of the portion soluble in ether and bicarbonate, led to the isolation of a second acid, $C_{11}H_{12}O_5$, m.p. 132–135° (decomp.). This acid, on treatment with hot dilute mineral acids, readily forms a neutral lactone, $C_{11}H_{10}O_4$, m.p. 173°, identical with deoxygladiolic acid, $C_{11}H_{10}O_4$, obtained by the reduction of gladiolic acid with iron powder and acetic acid. The acid, m.p. 132–135° (decomp.), may therefore be regarded as a dihydrogladiolic acid. Rather surprisingly, the bicarbonate-insoluble fraction of the same batch of crude gladiolic acid did not contain any of the phenol, $C_{10}H_{10}O_3$; in its place only deoxygladiolic acid, $C_{11}H_{10}O_4$, was obtained. In view of the ease with which dihydrogladiolic acid undergoes lactonization, deoxygladiolic acid may not be a true metabolic product of *P. gladioli*, but an artifact arising from the method of extraction and purification of the crude gladiolic acid.

Subsequently, dihydrogladiolic acid was shown to be identical with the dihydrogladiolic acid of Raistrick & Ross (1952); deoxygladiolic acid was identical with the dihydrogladiolide of the same workers.

Gladiolic acid, $C_{11}H_{10}O_5$, forms long colourless needles, m.p. 160°. It is optically inactive. The ultraviolet absorption curves (Fig. 1) show maxima at 3070 A. log ϵ 3·65 and 2690 A. log ϵ 4·04 in water, and 3430 A. log ϵ 2·82 and 2750 A. log ϵ 3·53 in 0·1 N-sodium hydroxide. Gladiolic acid contains one methoxyl group and titrates as a monobasic acid. It gives no colour with ferric chloride and the hydroxamic acid test for the carboxylic acid group (Feigl, 1947) is positive. The pK determined in the usual way from the potentiometric titration curve (Fig. 2) is 4·45 at 18°. Gladiolic acid is stable to hydrolysis by dilute mineral acids, but 1 mol. of carbon dioxide is liberated on heating with copper chromite in quinoline. On heating under reflux with methanol in the presence of dry HCl gas a methyl ester, $C_{12}H_{12}O_5$, is formed; esterification with ethanol and concentrated sulphuric acid gives an

ethyl ester, $C_{13}H_{14}O_5$. Gladiolic acid reacts with diazomethane, but the course of the reaction is not straightforward and nitrogen-containing material can be separated from the crude product. Gladiolic acid neither reduces Fehling's solution nor restores the colour to Schiff's reagent, but ammoniacal silver nitrate is reduced immediately in the cold. Neutral permanganate in acetone is rapidly decolorized on standing at room temperature, but bromine water is only decolorized very slowly on warming. On treatment with Brady's reagent gladiolic acid forms a dinitrophenylhydrazone, $C_{17}H_{14}O_8N_4$. With other carbonyl reagents such as phenylhydrazine and semicarbazide and with primary amines and diamines, the reactions are complex in nature, and will be the subject of a further communication, but in general, 1 mol. of gladiolic acid reacts with 2 mol. of the reagent or amine. The formation of an intense green colour with ammonia solution, sp.gr. 0·880 (Brian *et al.* 1946), has considerable value as a diagnostic test. Gladiolic acid also gives an interesting colour reaction with technical benzene and concentrated sulphuric acid, a deep-red ring being formed at the benzene/acid interface; this colour reaction is also given by many aromatic aldehydes. Gladiolic acid does not give the iodoform reaction, nor does it give a red colour with sodium nitroprusside and alkali. It is only very slowly oxidized by periodic acid, 42 % being recovered unchanged after 3 weeks at 30°. Gladiolic acid does not give the nitrochromic acid test for primary and secondary alcoholic —OH groups.

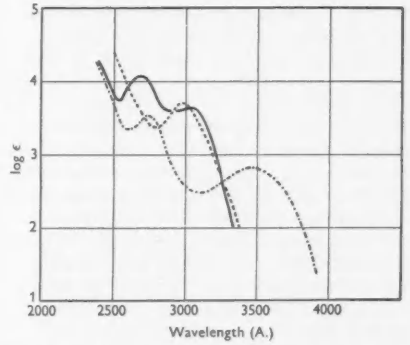


Fig. 1. Ultraviolet absorption spectra of gladiolic acid and *iso*gladiolic acid. ——, gladiolic acid in water; —·—·—, gladiolic acid on 0·1 N-NaOH; - - - - -, *iso*gladiolic acid in ethanol.

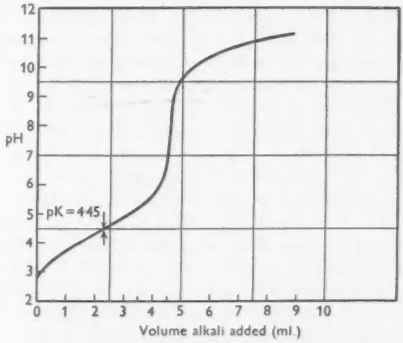


Fig. 2. Potentiometric titration of gladiolic acid at 18° with 0·1067 N-NaOH. Gladiolic acid approx. 5×10^{-3} M.

Three compounds have been isolated following the acetylation of gladiolic acid under different conditions. Acetylgladiolic acid, $C_{13}H_{12}O_6$, formed on heating gladiolic acid with acetic anhydride and glacial acetic acid for 1 hr. at 100° is no longer acidic, but is a neutral compound insoluble in cold sodium hydroxide and containing one methoxyl group. It no longer gives the colour reaction with ammonia solution given by gladiolic acid, but an intense red colour is produced with benzene and concentrated sulphuric acid. It reduces ammoniacal silver nitrate immediately in the cold, and contains a reactive carbonyl group, forming a dinitrophenylhydrazone, $C_{19}H_{16}O_9N_4$, and a semicarbazone, $C_{14}H_{15}O_6N_3$. Acetylgladiolic acid regenerates gladiolic acid on hydrolysis with hot dilute mineral acid. When gladiolic acid is heated with the acetic anhydride/acetic acid acetylating mixture at 100° for some hours a second neutral product, $C_{17}H_{18}O_9$, is obtained. It contains three acetyl groups and may be regarded as the triacetate of a hypothetical gladiolic acid hydrate, $C_{11}H_{12}O_6$. The triacetyl compound does not reduce ammoniacal silver nitrate and shows no carbonyl reactivity, although on prolonged heating in aqueous solution with semicarbazide acetate, acetylgladiolic acid semicarbazone is formed in low yield. The triacetyl compound is also formed when acetylgladiolic acid is heated at 100° with acetic anhydride in the presence of concentrated sulphuric acid. Under more drastic conditions of acetylation, namely, heating under reflux with acetic anhydride and fused sodium acetate, gladiolic acid is converted into an unsaturated monobasic acid, $C_{15}H_{14}O_7$, which contains one methoxyl group and one acetyl group which is liberated as acetic acid on acid hydrolysis. The acid, $C_{15}H_{14}O_7$, is non-ketonic and does not reduce ammoniacal silver nitrate.

Gladiolic acid is sensitive to alkali and, on boiling with 3 N-sodium hydroxide for some minutes or on standing with 2 N-sodium hydroxide for 24 hr. at room temperature, is transformed quantitatively into *iso*gladiolic acid, $C_{11}H_{10}O_5$, m.p. 234°. However, gladiolic acid is unaffected by solution in 0·1 N-sodium hydroxide for 30 min. at 25° and on acidi-

fication is recovered unchanged. *iso*Gladiolic acid contains one methoxyl group and gives no colour with ferric chloride. It titrates as a monobasic acid, but lactone titration discloses the presence of a second acidic grouping. Although the lactone ring is readily opened, for example on standing with excess 0·1 N alkali at 20°, *iso*gladiolic acid is recovered unchanged on acidification even when this is carried out carefully with ice-cold solutions. On heating *iso*gladiolic acid with copper chromite in quinoline at 220°, 1 mol. of carbon dioxide is evolved and a phenolic compound, $C_9H_8O_3$, is obtained in poor yield. This phenol, $C_9H_8O_3$, gives no colour with ferric chloride; it contains a lactone ring and no methoxyl group, and *iso*gladiolic acid appears to have undergone demethylation in addition to the loss of the carboxyl group.

*iso*Gladiolic acid does not give a coloration with ammonia solution, sp.gr. 0·880, or with benzene and concentrated sulphuric acid. In marked contrast to gladiolic acid, *iso*gladiolic acid does not form a precipitate with Brady's reagent and is recovered unchanged even after heating under reflux for 6 hr. with acetic anhydride and fused sodium acetate. On esterification under Fischer-Speier conditions, *iso*gladiolic acid forms a neutral methyl ester, $C_{12}H_{12}O_5$, which like the parent acid shows no carbonyl reactivity. The ultraviolet absorption curve, $\lambda_{max.}$ 2980 A., log ϵ 3·72 in ethanol (Fig. 1) supports the conclusion that a molecular rearrangement is involved in the formation of *iso*gladiolic acid.

As might be expected, *iso*gladiolic acid is also formed by alkaline hydrolysis of acetylgladiolic acid, triacetylgladiolic acid hydrate, methyl gladiolate and ethyl gladiolate.

Demethylation of *iso*gladiolic acid with hydriodic acid or better with hydrobromic acid gives nor*iso*gladiolic acid, $C_{10}H_8O_5$, a phenolic compound giving a deep reddish-purple colour with ferric chloride. nor*iso*Gladiolic acid couples immediately with diazotized *p*-nitraniline but only very slowly with diazotized aniline. The Millon reaction is negative. In the test with 2:6-dibromoquinone chloroimide (Gibbs reaction: Gibbs, 1927), a faint blue colour develops slowly. Nor*iso*gladiolic acid is also formed, together with much tarry product, when gladiolic acid is heated under reflux with hydrobromic acid.

Like acetylgladiolic acid, methyl gladiolate does not give a green coloration with ammonia solution, but gives a red colour with benzene and concentrated sulphuric acid and reduces ammoniacal silver nitrate. With Brady's reagent, methyl gladiolate gives an immediate precipitate of the dinitrophenylhydrazone, $C_{18}H_{16}O_8N_4$. On reaction with phenylhydrazine hydrochloride in aqueous solution the phenylhydrazone, $C_{18}H_{18}O_4N_2$, is formed, and on treatment with acetic anhydride and concentrated sulphuric acid at 0°, the diacetate, $C_{16}H_{18}O_8$, of a hydrate of methyl gladiolate is produced. The diacetyl compound does not immediately form a precipitate with Brady's reagent, but on standing methyl gladiolate dinitrophenylhydrazone separates. It reduces ammoniacal silver nitrate on warming but more slowly than methyl gladiolate.

Ethyl gladiolate forms a similar series of derivatives including a semicarbazone, $C_{14}H_{17}O_5N_3$, and a diacetate, $C_{17}H_{20}O_8$, corresponding to the diacetate, $C_{16}H_{18}O_8$, of methyl gladiolate. Ethyl gladiolate slowly restores the colour to Schiff's reagent.

On reduction of gladiolic acid with iron powder and acetic acid the main product is deoxygladiolic acid, $C_{11}H_{10}O_4$, m.p. 173°, which is no longer an acidic substance. An acidic compound is also produced but in an amount too small for adequate characterization. Deoxygladiolic acid contains a lactone ring and is identical, by mixed melting points and comparison of infrared absorption spectra, both with the lactone, $C_{11}H_{10}O_4$, obtained from dihydrogladiolic acid isolated from the crude gladiolic acid, and also with the dihydrogladiolide of Raistrick & Ross (1952). Deoxygladiolic acid contains one methoxyl group, reduces ammoniacal silver nitrate and shows carbonyl reactivity forming an immediate precipitate of the dinitrophenylhydrazone, $C_{17}H_{14}O_7N_4$, with Brady's reagent. Cautious demethylation with hydrobromic acid yields nordeoxygladiolic acid, $C_{10}H_8O_4$, which gives a deep reddish-violet colour with ferric chloride. *iso*-Gladiolic acid, $C_{11}H_{10}O_5$, is formed on oxidation of deoxygladiolic acid with acid permanganate.

The main product of the Clemmensen reduction of gladiolic acid is a neutral lactone, $C_{11}H_{12}O_3$, which contains one methoxyl group and is non-ketonic. Demethylation of this substance with hydrobromic acid yields a phenolic lactone, $C_{10}H_{10}O_3$, m.p. 158°, identical with the phenolic substance, $C_{10}H_{10}O_3$, present in crude gladiolic acid. It gives an intense blue colour with ferric chloride. It does not couple with diazotized *p*-nitraniline and the Millon and Gibbs reactions are negative. A second neutral substance having the probable molecular formula, $C_{22}H_{20}O_6$, resulting from the condensation of two molecules of gladiolic acid, is present in the tarry by-products of the Clemmensen reduction and can be isolated therefrom by trituration with ethanol, in which it is sparingly soluble, followed by vacuum sublimation.

Oxidation of gladiolic acid, $C_{11}H_{10}O_5$, with permanganate gives rise to a series of acidic products according to the conditions employed. Substance *A*, obtained in good yield by the action of 1 % potassium permanganate solution equivalent to one oxygen atom per molecule of gladiolic acid in sodium carbonate at room temperature, is a dibasic acid, $C_{11}H_{10}O_6$. It contains one methoxyl group and

gives no coloration with ferric chloride. The fluorescein and phenolphthalein colour tests for an *o*-phthalic acid are negative. It does not reduce ammoniacal silver nitrate, but nevertheless shows carbonyl reactivity forming a dinitrophenylhydrazone, $C_{17}H_{14}O_9N_4$, a semicarbazone, $C_{12}H_{13}O_6N_3$, which titrates as a dibasic acid, and a monobasic anhydrophenylhydrazone, $C_{17}H_{14}O_4N_2$. In contrast to gladiolic acid, substance *A* is unaffected by boiling 3N-sodium hydroxide. It is recovered unchanged after standing 12 hr. with 40% aqueous potassium hydroxide at room temperature. On mild acetylation with acetic anhydride and glacial acetic acid, substance *A* forms an acetate, $C_{13}H_{12}O_7$, which titrates as a monobasic acid and shows no ketonic properties. This acetate is dimorphous, forming needles with m.p. 199° from dilute acetic acid and prisms, m.p. 213°, from aqueous ethanol, and is also formed in the oxidation of acetylgladiolic acid by permanganate in acetone solution.

Oxidation of gladiolic acid with excess permanganate in sodium carbonate at room temperature or further oxidation of substance *A* under the same conditions gives substance *B*, $C_{11}H_{10}O_7$, m.p. 162°, a tribasic acid, which contains one methoxyl group, forms a trimethyl ester, $C_{14}H_{16}O_7$, gives no colour with ferric chloride and is non-ketonic. Substance *B* gives strongly positive results in the usual tests for an *o*-phthalic acid.

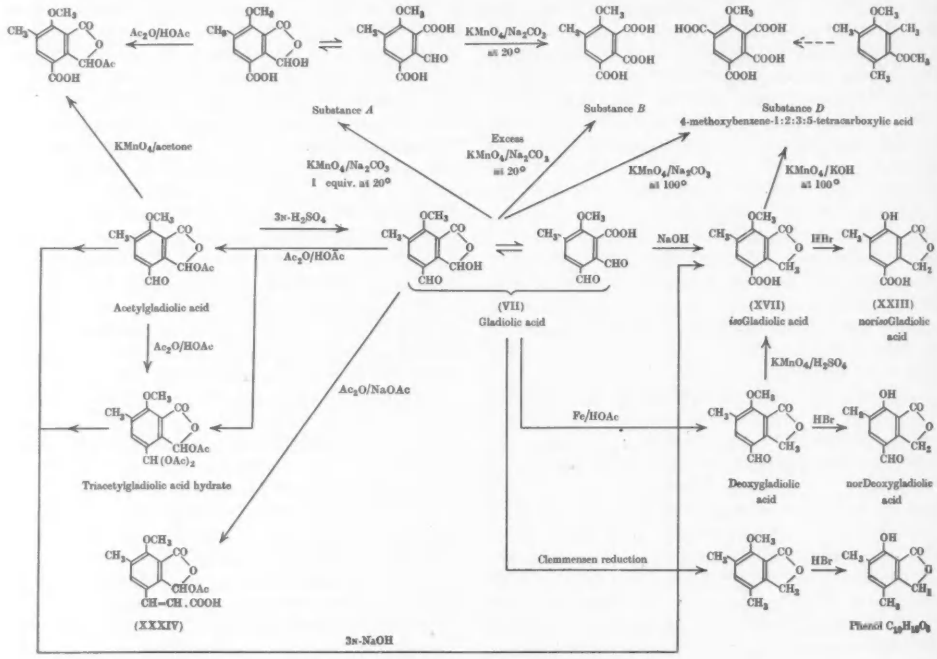
Two products have been obtained when gladiolic acid is oxidized in alkaline solution with excess boiling potassium permanganate, a tribasic acid substance *C*, $C_{10}H_6O_7$, m.p. 160–165° (decomp.), and a tetrabasic acid substance *D*, $C_{11}H_8O_9$. Crystallized from ethyl acetate substance *D* has m.p. 175–180° (decomp.) resetting about 220° and remelting at 259° (decomp.): crystallized from dilute hydrochloric acid the initial melting point is somewhat higher (190° decomp.), but the melt then resets and remelts at 259° (decomp.) as already described. Substance *C* was obtained, although in poor yield, in one experiment only, in which 5% potassium permanganate was added to gladiolic acid in boiling sodium carbonate, and attempts to repeat this preparation have only given traces of material. Unfortunately, it has not been possible to accumulate sufficient material for the preparation of a derivative. When the oxidation is carried out initially at room temperature, and then continued, when the rate of uptake of permanganate has slowed, by heating under reflux, the main product is substance *D* and only traces of substance *C* are obtained. Substance *D* is the sole product both of the oxidation of *iso*gladiolic acid by permanganate in boiling potassium hydroxide and of gladiolic acid in the same medium. Undoubtedly under these conditions gladiolic acid is first converted into *iso*gladiolic acid. The melting point of substance *B* is not depressed by admixture with substance *C* or with substance *D*, but the three acids may be recognized by their infrared absorption spectra which show characteristic bands.

Whereas substance *C* is readily soluble in ether and can be sublimed at 150°/10^{-4} mm., substance *D* is only slightly soluble in ether and does not sublime at 160°/10^{-4} mm.; hence the separation of the two acids when they occur together is a relatively simple matter. In the original oxidation experiment in which permanganate was added to gladiolic acid in boiling sodium carbonate, the resulting aqueous solution was evaporated to dryness with concentrated hydrochloric acid to avoid the possible isolation of mono-sodium or mono-potassium salts and the dry solid residue extracted with ether. It is not surprising, therefore, that only substance *C* and no substance *D* was obtained. Both substances *C* and *D* give positive reactions in the tests for *o*-phthalic acids, and it is reasonable to regard substance *C* as decarboxy substance *D*. Substance *D* is not decarboxylated by heating at 150° *in vacuo* and it follows that the decarboxylation must take place either during the early stages of the oxidation or directly as a result of the method adopted for working up the reaction mixture. For decarboxylation to occur as a result of either of these possibilities is unusual; however, cases have been reported in the literature where aromatic polycarboxylic acids undergo decarboxylation on heating with concentrated mineral acids (Baeyer, 1873), and this provides a possible explanation for the formation of substance *C*.

Substance *D* gives no colour with ferric chloride. It contains one methoxyl group and forms a liquid tetramethyl ester, $C_{15}H_{16}O_9$. It is clearly a methoxybenzenetetracarboxylic acid and on demethylation with hydrobromic acid gives the corresponding hydroxybenzenetetracarboxylic acid, $C_{10}H_6O_9$, m.p. 302°. The latter compound gives a red colour with ferric chloride, and on methylation with ethereal diazomethane in methanol the tetramethyl ester of substance *D* is produced. The hydroxybenzenetetracarboxylic acid couples very slowly with diazotized amines, but both the Millon and Gibbs reactions are negative. Although, prior to this investigation, none of the three possible hydroxybenzenetetracarboxylic acids or their methyl ethers had actually been synthesized, one hydroxybenzenetetracarboxylic acid and one methoxybenzenetetracarboxylic acid are reported in the literature as having been obtained by the degradation of certain natural products, the structures of which are known with such certainty as to leave little doubt as to the configuration of the degradation products. Thus Dimroth & Goldschmidt (1913) obtained 5-hydroxybenzene-1:2:3:4-tetracarboxylic

acid, m.p. 212°, with anhydride formation by the degradation of laccaic acid, and Nikuni (1941) isolated 3-methoxybenzene-1·2:4:5-tetracarboxylic acid, m.p. 250–251° (decomp.), by oxidation of dimethyl-α-sorijenin. It seemed probable, therefore, that substance *D* was in fact 4-methoxybenzene-1:2:3:5-tetracarboxylic acid, and this conclusion was supported by the negative Millon and Gibbs reactions given by the demethylated acid.

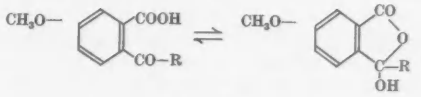
Accordingly, 4-methoxybenzene-1:2:3:5-tetracarboxylic acid has been synthesized as follows: the Friedel-Crafts reaction between mesityl methyl ether and acetyl chloride in the presence of 1·5 moles of anhydrous aluminium chloride was carried out according to Auwers & Mauss (1928), and the crude product separated into phenolic and neutral fractions by extraction with sodium hydroxide. Auwers & Mauss obtained a 20% yield of 3-hydroxy-2:4:6-trimethylacetophenone, but in their hands the neutral fraction consisted of only a little yellow oil in addition to unchanged mesityl methyl ether. In the present investigation the phenolic fraction consisted of only a trace of 3-hydroxy-2:4:6-trimethylacetophenone, whereas the neutral fraction, a yellow oil, b.p. 200–275°, contained, in addition to unreacted mesityl methyl ether, mesityl acetate, 3-methoxy-2:4:6-trimethylacetophenone and 3-acetoxy-2:4:6-trimethylacetophenone. Very similar results were obtained using 3 moles of aluminium chloride and acetic anhydride, in place of acetyl chloride. The crude neutral product was separated by distillation into two arbitrary fractions, b.p. 200–240°, and 250–275° respectively, and each fraction separately hydrolysed with boiling 3N-sodium hydroxide to remove the acetates. 3-Methoxy-2:4:6-trimethylacetophenone, b.p. 268–270°, was obtained from the high-boiling fraction; acidification of the alkaline mother liquors yielded 3-hydroxy-2:4:6-trimethylacetophenone. Oxidation



of 3-methoxy-2:4:6-trimethylacetophenone, first with boiling permanganate in alkaline solution and then with acid permanganate to bring about decarboxylation of the resultant phthalonic acid (Posternak, 1940) gives in good yield, 4-methoxybenzene-1:2:3:5-tetracarboxylic acid, identical by mixed melting-point determinations and comparison of the infrared absorption spectra with substance *D*, the methoxybenzenetetracarboxylic, acid from gladiolic acid. 4-Hydroxybenzene-1:2:3:5-tetracarboxylic acid, obtained on demethylation of the synthetic material with hydrobromic acid, is identical with the hydroxybenzenetetracarboxylic acid, m.p. 302°, obtained by demethylation of substance *D*.

DISCUSSION

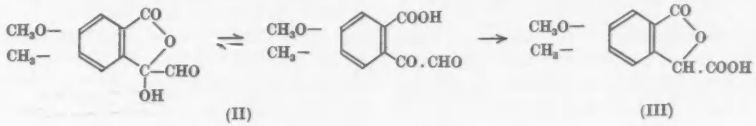
Isomeric structural formulae for gladiolic acid are readily deduced from the chemical work described above. Many of the compounds obtained from gladiolic acid, in particular the products of oxidation and the demethylated reduction products, are clearly aromatic in character. The presence of a benzene nucleus in gladiolic acid itself is indicated by the stability of the compound towards bromine, and is supported by the ultraviolet absorption spectrum which is typical of a substituted aromatic compound in which benzenoid and carbonyl chromophores are conjugated. Gladiolic acid, $C_{11}H_{10}O_5$, is a monobasic carboxylic acid. It contains a carbonyl group as shown by the formation of a dinitrophenylhydrazone, and this must be a formyl group, since on mild oxidation with permanganate, the dibasic acid substance *A*, $C_{11}H_{10}O_6$, is produced and the reducing properties of gladiolic acid are eliminated. The presence of a tautomeric system is most easily seen from an examination of the chemistry of substance *A*. Of the six oxygen atoms in this compound, four occur in the two —COOH groups, one of which was originally present in the gladiolic acid molecule and the second formed during the oxidation. The fifth oxygen atom is contained in a $—OCH_3$ group and the sixth is present as a $\rangle C{=}O$ group as shown by the formation of the semicarbazone which titrates as a dibasic acid. Nevertheless, the presence of a hydroxyl group in substance *A* is indicated by the formation on acetylation under mild conditions of a monobasic monoacetate, $C_{13}H_{12}O_7$, and this can be explained if substance *A* contains a γ- or δ-keto acid structure capable of showing keto-lactol tautomerism. Kühn-Roth oxidations on gladiolic acid and its derivatives suggest the presence of 1*C*-CH_3 group and if this is admitted, substance *A* must be a γ-keto acid and contains the partial structure (I). Of the five oxygen atoms in gladiolic acid, four are present in the methoxyl substituted keto-acid structure I; the fifth is contained in a formyl group which cannot be the carbonyl group involved in the tautomeric system I, since the neutral acetylgladiolic acid, $C_{13}H_{12}O_6$, which is derived from the lactol form retains the carbonyl reactivity and reducing properties of gladiolic acid.



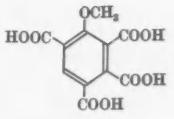
(I)

*iso*Gladiolic acid, $C_{11}H_{10}O_5$, on the other hand, shows no reducing properties or carbonyl reactivity and the presence of a hydroxyl group cannot be detected; the five oxygen atoms are in fact contained in one $—OCH_3$ group, one carboxyl group and one lactone ring. It may be concluded, therefore, that the molecular rearrangement concerned in the formation of *iso*gladiolic acid involves both the formyl group and the carbonyl group of the keto-lactol system of gladiolic acid.

All the oxygen and carbon atoms in gladiolic acid have been accounted for in the various groupings detected and it only remains to determine how these groupings are oriented with respect to each other. Thus the additional nuclear substituents in the partial structure I may be $—CH_3$, —CHO, or $—CH_3$ and —CHO, depending on whether R is —CHO, $—CH_3$, or H respectively.



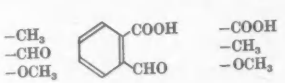
(II) (III)



(IV)

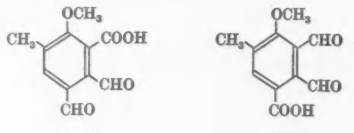
Gladiolic acid gives neither the iodoform reaction, nor the nitroprusside test for the $CH_3.CO—$ group and it may be concluded that this grouping is absent. Following the original oxidation experiment which led to the isolation of the methoxybenzenetricarboxylic acid, substance *C*, it was concluded that gladiolic acid contained a benzene ring with a methoxyl substituent and three carbon side chains, and in a preliminary communication (Brian *et al.* 1946) a 2-carboxyphenylglyoxal structure II was suggested as being consistent with the chemistry of gladiolic acid as then known. On this structure the formation of *iso*gladiolic acid from gladiolic acid in alkali was visualized as involving the normal rearrangement, under such conditions, of the phenyl

glyoxal portion of the molecule to the corresponding mandelic acid followed by lactonization to the phthalide-3-carboxylic acid (III). Subsequently, it was discovered that gladiolic acid did not undergo

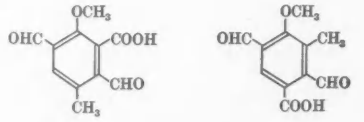


(V) (VI)

fission with periodate with the formation of a methylmethoxyphthalic acid as might have been expected on structure II; moreover, when the oxidation with permanganate was re-examined the



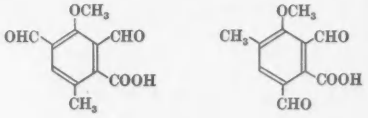
(VII) (VIII)



(IX) (X)

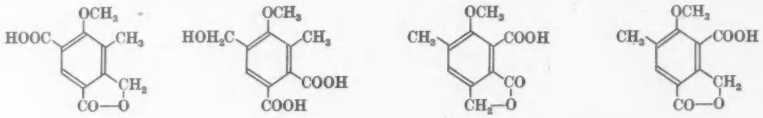
isolation of a tetracarboxylic acid, shown by synthesis to be 4-methoxybenzene-1:2:3:5-tetracarboxylic acid (IV), from both gladiolic acid and *iso*gladiolic acid clearly excluded structures II and III

The well known rearrangement of *o*-phthalaldehyde under alkaline conditions to give hydroxymethylbenzoic acid usually isolated as its lactone, phthalide, provides a ready explanation of the formation of *iso*gladiolic acid. VII and VIII are therefore seen to be the more probable structures for gladiolic acid. (For simplicity, only the open, keto, structures will be considered in the arguments which follow.)

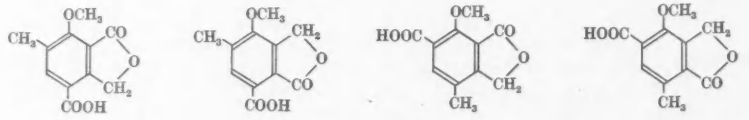


(XI) (XII)

Nevertheless structures IX and X, in which the formyl substituents are 1:4, and XI and XII, in which they are 1:3, cannot at this stage be completely disregarded as *iso*gladiolic acid can theoretically be derived from them by a type of crossed Cannizzaro reaction followed by lactonization. For example, by this reaction sequence, X can give rise to XIII, and IX and XI to XIX and XX respectively. A second product, namely the substituted phthalic acid XIV, might also be expected from X, and similar considerations apply to crossed Cannizzaro reactions with IX and XI. The isolation of a phthalic acid from gladiolic acid under the influence of alkali has not been realized experimentally: however, the absence of a second product cannot be used as an argument against the *iso*- and tere-phthalaldehyde structures since the *o*-phthalaldehyde structures VII and VIII are each capable of rearrangement to three products, the phthalide carboxylic acids



(XIII) (XIV) (XV) (XVI)



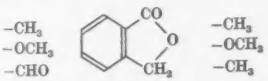
(XVII) (XVIII) (XIX) (XX)

for these compounds. The isolation of the tetracarboxylic acid in fact admits of no other arrangement but V for gladiolic acid, comprising (in the open form) a benzene nucleus with methyl, methoxyl, carboxyl and two formyl substituents, and VI for *iso*gladiolic acid, namely a phthalide ring with nuclear methyl, methoxyl and carboxyl substituents.

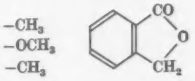
XV–XVII, and XVI–XVIII respectively. Under the experimental conditions employed, a mixture of phthalide carboxylic acids is not obtained: *iso*gladiolic acid is a pure substance and the only product of the reaction. A possible explanation for this observation is advanced in the closing paragraphs of the discussion.

Returning to a consideration of the possible structures for gladiolic acid, XII can be disregarded, as the dicarboxylic acid substance *A* is not an *o*-phthalic acid. Some support for the *o*-phthalaldehyde structures VII and VIII comes from the colour reaction of gladiolic acid with ammonia; *o*-phthalaldehyde, but not *iso*- or tere-phthalaldehyde, gives a bluish-green colour on acidification of an ammoniacal solution with dilute acetic acid. The final decision between the isomeric structures VII–XI comes from a consideration of the structures of *iso*gladiolic acid and also of the reduction products of gladiolic acid, deoxygladiolic acid, $C_{11}H_{10}O_4$, obtained by the action of iron powder and acetic acid, and the lactone, $C_{11}H_{12}O_3$, obtained by Clemmensen's method.

Deoxygladiolic acid retains the carbonyl reactivity and reducing properties and hence the reactive formyl group of gladiolic acid. Apparently only the phthalaldehydic acid portion of the molecule is affected by the reduction; either reduction of the formyl group in the tautomeric system (I, R = H) occurs preferentially and is followed by lactonization or more probably, gladiolic acid is reduced in the lactol form directly to the corresponding phthalide. Deoxygladiolic acid then has the partial structure XXI.



(XXI)



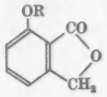
(XXII)

Reduction of the 3-hydroxyphthalide system to phthalide occurs in the Clemmensen reduction, but in addition the formyl group is reduced to —CH_3, and the compound $C_{11}H_{12}O_3$ has the partial structure XXII.

Oxidation of deoxygladiolic acid in dilute mineral acid gives *iso*gladiolic acid and if we assume, as seems reasonable, that the phthalide ring remains intact under these conditions, then the reactive formyl group of gladiolic acid must correspond to the free carboxyl group in *iso*gladiolic acid. Consequently, structures XV and XVI for *iso*gladiolic acid can only come from VIII, and XVII and XVIII can only come from VII. (As will be seen below, evidence obtained at one stage of the investigation threw some doubt on the validity of the above assumption and on the deductions made.)

All three substances, *iso*gladiolic acid, deoxygladiolic acid and the Clemmensen reduction product $C_{11}H_{12}O_3$ can be demethylated to phenolic substances, but little information on the orientation of these compounds can be obtained from the Millon and Gibbs tests or from coupling reactions which are negative, except in the case of nor*iso*gladiolic acid. The positive coupling with diazotized *p*-nitraniline shown by nor*iso*gladiolic acid no doubt involves displacement of the carboxyl group. Nor*iso*gladiolic acid gives an intense reddish-purple colour with ferric chloride showing that the hydroxyl substituent must be ortho to a carbonyl group present either in the carboxylic acid residue or in the phthalide ring and eliminating structure XVIII for *iso*gladiolic acid. Further information on the orientation of the substituents in nor*iso*gladiolic acid can be obtained from the infrared spectrum.

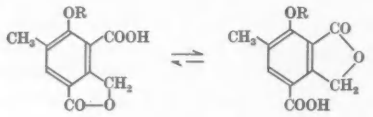
Recent work on the correlation of absorption frequency with structure for the >C=O stretching vibration has been summarized by Grove & Willis (1951). Chelation in *o*-hydroxycarbonyl compounds results in very large displacements of the characteristic absorption frequencies of both the >C=O and —OH groups (Gordy, 1940; Martin, 1950; Hunsberger, 1950). It follows that these displacements may be used to detect chelation in compounds of unknown orientation which contain a carbonyl and a hydroxyl group, and the method is more reliable if the corresponding methyl ether is also available for purposes of comparison. If two >C=O groups of different types each having its own characteristic absorption band are present in the same molecule, the method may be extended to detecting which of the two >C=O groups is chelated. *iso*Gladiolic acid shows two bands in the double bond-stretching region at 1758 and 1688 cm.$^{-1}$ due to phthalide ring >C=O and aromatic carboxylic acid >C=O respectively (Grove & Willis, 1951). Nor*iso*gladiolic acid shows only a single broad absorption band with its peak at 1695 cm.$^{-1}$ and a definite shoulder at 1727 cm.$^{-1}$. The —OH absorption frequency of nor*iso*gladiolic acid is so low that it is masked by the C—H absorption of the 'Nujol' medium, and it may be deduced that the compound contains a chelate ring and that chelation involves the >C=O group of the phthalide ring and not the >C=O group of the carboxyl group. Nor*iso*gladiolic acid must therefore contain the partial structure XXIII



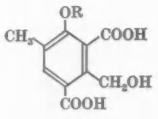
(XXIII)

(R = H), and XIII, XV and XX for *iso*gladiolic acid are thereby eliminated as they cannot, under the experimental conditions employed in the demethylation, give a nor*iso*gladiolic acid with structure XXIII (R = H).

The elimination of XIII and XX for *iso*gladiolic acids disposes of X and XI respectively as possible structures for gladiolic acid. Now the phenol, $C_9H_8O_3$, obtained by decarboxylation of *iso*gladiolic acid with copper chromite in quinoline gives no colour with ferric chloride. The phenol derived from structure XIX for *iso*gladiolic acid by decarboxylation and demethylation should give an intense ferric reaction, and structures XIX for *iso*gladiolic acid and IX for gladiolic acid may therefore be excluded. Of the two remaining structures for *iso*gladiolic acid, XVI and XVII, the absence of a ferric reaction in the phenol, $C_9H_8O_3$, clearly favours the salicylic acid structure XVI; on the other hand, the partial structure XXIII deduced above for nor*iso*gladiolic acid is derived from XVII. It must be borne in mind that during the demethylation of *iso*gladiolic acid under acid conditions with hydrobromic acid, a rearrangement of type XXIV, although unlikely, is not impossible. A rearrangement of this type is of course possible in the decarboxylation experiment and indeed is more likely than under the acid conditions of the demethylation. Therefore, if we assume



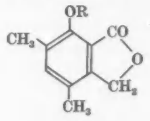
(XXIV)



(XXV)

that there is no rearrangement of type XXIV involved in the formation of nor*iso*gladiolic acid, *iso*gladiolic acid must be *7-methoxy-6-methylphthalide 4-carboxylic acid* XVII. Although this line of reasoning appears to exclude VIII and establishes gladiolic acid as VII, *4-methoxy-5-methyl-o-phthalaldehyde 3-carboxylic acid* (McGowan, 1950), the assumption involved, namely that there is no rearrangement of type XXIV during the oxidation of deoxygladiolic acid to *iso*gladiolic acid, is precisely that used to exclude XVI as a possible structure for *iso*gladiolic acid following the establishment of nor*iso*gladiolic acid as containing XXIII (R = H). It is relevant to point out here that the open structure XXV (R = CH_3) must be present as the sodium salt in alkaline solution following the rearrangement of gladiolic acid, and that on acidification this closes preferentially to give only one product.

In view of the ambiguity involved, further evidence was sought by the examination of the phenol, $C_{10}H_{10}O_3$, obtained by demethylation of the Clemmensen reduction product, $C_{11}H_{12}O_3$. This phenol gives a blue colour with ferric chloride in contrast to nor*iso*gladiolic acid which gives a reddish purple. In the infrared spectrum, the —OH and >C=O groups absorb at 3378 cm.$^{-1}$ (2·96 μ.) and 1742 cm.$^{-1}$ respectively, the normal positions for non-chelated phenolic hydroxyl and phthalide ring >C=O respectively. In the methyl ether, the phthalide ring >C=O band has its maximum at 1745 cm.$^{-1}$. It was concluded, therefore, that the phenol had in fact the structure XXVII (R = H) and not XXVI (R = H) corresponding to VII. On this basis it was concluded that gladiolic acid was



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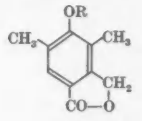
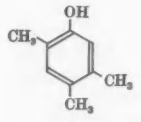


(XXVII)



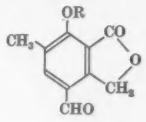
(XXVIII)

VIII, 3-methoxy-4-methyl-*o*-phthalaldehyde 6-carboxylic acid and that a rearrangement of type XXIV did take place either during the oxidation of deoxygladiolic acid to *iso*gladiolic acid or during the demethylation of *iso*gladiolic acid. Moreover, the absence of a ferric reaction in the phenol $C_9H_8O_3$ supported the conclusions that *iso*gladiolic acid had the structure XVI and that a rearrangement occurred during the demethylation of *iso*gladiolic acid.

At this point in the investigation the information that the phenol $C_{10}H_{10}O_3$, which can also be obtained by Clemmensen reduction of dihydrogladiolic acid and demethylation of the reduction product (Raistrick & Ross, 1952), gave ψ-cumenol (2:4:5-trimethylphenol) XXVIII on treatment with hydriodic acid and red phosphorus, was communicated to the present author by Prof. H. Raistrick, F.R.S. In this case there is no possibility of a rearrangement of type XXIV and this evidence, subject to the proviso that there is no migration of methyl groups round the benzene ring, establishes the structure of the phenol $C_{10}H_{10}O_3$ as 7-hydroxy-4:6-dimethylphthalide XXVI (R = H). It follows that gladiolic acid has structure VII, 4-methoxy-5-

methyl-*o*-phthaldehyde 3-carboxylic acid, a structure which is certainly favoured by the bulk of the chemical evidence and is now believed to be correct.

Additional evidence in support of this structure comes from an examination of the infrared spectrum of nordeoxygladiolic acid. Nordeoxygladiolic acid gives an intense reddish-purple colour with ferric chloride which cannot be used to distinguish between XXIX (R = H) (derived from VII) and XXX



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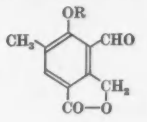

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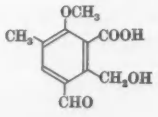

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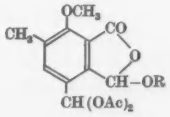
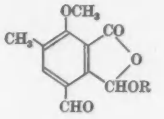


(XXXII)

(R = H) (derived from VIII), both salicylaldehyde and salicylic acid giving very similar colours with ferric chloride. However, the infrared spectrum shows two bands due to >C=O groups at 1733 and 1688 cm.$^{-1}$ compared with 1758 and 1695 cm.$^{-1}$ in deoxygladiolic acid. As in the case of nor*iso*-gladiolic acid, the shift of 25 cm.$^{-1}$ shows that the >C=O group in the phthalide ring is chelated and XXIX (R = H) is therefore preferred to XXX (R = H). In this series there is no possibility of molecular rearrangement and deoxygladiolic acid must be XXIX (R = CH_3). It follows that gladiolic acid has the structure VII.

The failure of the infrared method in the case of the phenol $C_{10}H_{10}O_3$ is surprising, and is at present under investigation in these laboratories. There is no reason for doubting the validity of the method in those cases where displacements of the bands are actually observed.

The structures of the majority of the derivatives and degradation products of gladiolic acid follow immediately the orientation of the substituents has been established. Deoxygladiolic acid and dihydrogladiolic acid are 4-formyl-7-methoxy-6-methylphthalide XXIX (R = CH_3) and 3-formyl-2-hydroxymethyl-6-methoxy-5-methylbenzoic acid XXXI respectively, as conclusively and independently established by Raistrick & Ross. It seems more reasonable to suppose that the partial structure XXIII (R = CH_3 or H) persists throughout the sequence deoxygladiolic acid → *iso*gladiolic acid → nor*iso*gladiolic acid and that *iso*gladiolic acid is 7-methoxy-6-methylphthalide 4-carboxylic acid XVII. Moreover, the methyl*iso*gladiolate must be derived from XVII since ortho disubstituted benzoic acids do not readily form normal esters under Fischer conditions. However, the alternative structure XVI, 5-methoxy-6-methylphthalide 4-carboxylic acid cannot be completely excluded in view of the results obtained in the decarboxylation experiment.



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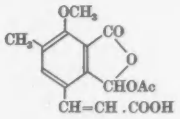
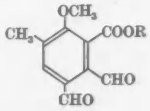


(XXXIV)

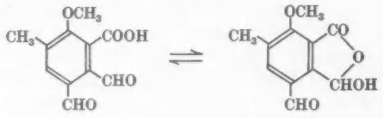


(XXXV)

Of the acetylation products of gladiolic acid, acetylgladiolic acid has the structure XXXIII (R = Ac) and the triacetyl compound, XXXII (R = Ac), the diacetate of the hydrated form of acetylgladiolic acid. Compounds of similar structure are formed by many reactive aldehydes and are described in the literature (e.g. *o*-phthalaldehyde tetra-acetate and phenylglyoxal diacetate). The unsaturated monobasic acid, $C_{15}H_{14}O_7$, XXIV, which contains one acetyl group is formed by Perkin condensation between the reactive formyl group of acetylgladiolic acid and acetic anhydride. The formation by methyl and ethyl gladiolate of diacetyl compounds, XXXII, which show no carbonyl reactions, suggests that these esters are pseudo esters of structure XXXIII (R = CH_3 and C_2H_5 respectively), rather than normal esters (XXXV) which might be expected to form either tetra-acetates or aldehydic diacetates. Conclusive spectroscopic evidence for the structures of these esters will be reported elsewhere. It may be noted that although ortho disubstituted benzoic acids do not form normal esters under Fischer conditions, their ability to form pseudo esters, where this is possible, is not greatly inhibited (Newman & McCleary, 1941; Newman & Lord, 1944).

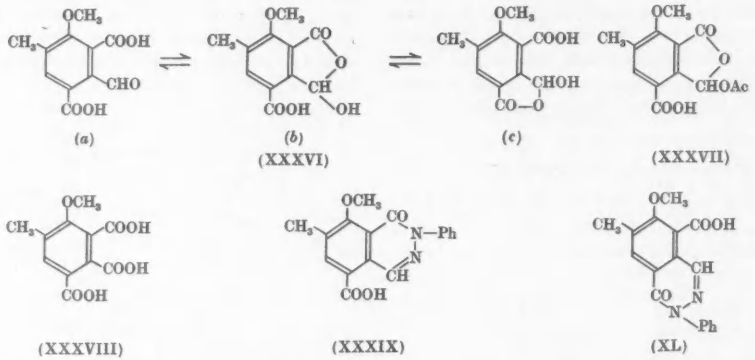
Of the oxidation products, substance *B* is 4-methoxy-5-methylbenzene-1:2:3-tricarboxylic acid, XXXVIII. Substance *A* has the tautomeric structure XXXVI, the monobasic monoacetate, $C_{13}H_{12}O_7$, XXXVII, being derived from the lactol form XXXVI*b* since it is also formed on oxidation of acetylgladiolic acid XXXIII (R = Ac). A derivative of the lactol form XXXVI*c* has not been obtained.

The precise structure of the monobasic anhydrophenylhydrazone which is now seen to be a methoxymethylphthalazone carboxylic acid (XXXIX or XL), derived from the keto form XXXVI*a* of substance *A*, is uncertain. The failure of substance *A* to undergo the Cannizzaro reaction is consistent with XXXVI*a*; it is well known that ortho disubstituted benzaldehydes do not readily undergo this reaction owing to steric hindrance.



(VII)

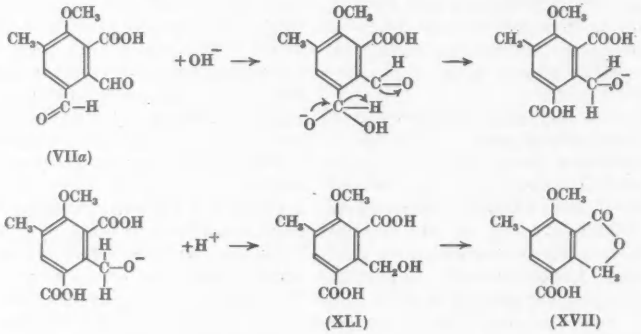
Keto-lactol tautomerism in gladiolic acid (VII) is shown by the rearrangement of the keto form VII*a* to *iso*gladiolic acid and by the formation of the neutral acetylgladiolic acid and the ψ esters derived from the lactol form VII*b*, 4-formyl-3-hydroxy-7-methoxy-6-methylphthalide. It is most clearly demonstrated by the differences in the ultraviolet absorption spectra (Fig. 1) of the compound in water and in 0·1N-sodium hydroxide in which gladiolic acid is stable.



(XXXVIII) (XXXIX) (XL)

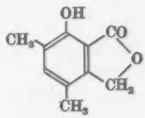
One further topic of gladiolic acid chemistry which remains to be discussed is the formation of a single product, *iso*gladiolic acid, on treatment of gladiolic acid with alkali. If, in the rearrangement of *o*-phthaldehyde, we assume a mechanism involving an intramolecular transfer of H^- similar to that recently advanced (Alexander, 1947; Doering *et al.* 1948) for the rearrangement of phenylglyoxal to mandelic acid, then the formyl group which undergoes the initial attack by the hydroxyl ion must become a carboxyl group in the resulting hydroxymethylbenzoic acid.

In the gladiolic acid molecule this initial attack is most likely to take place on the more reactive of the two formyl groups, namely that in position 1, and hence only one substituted hydroxymethylbenzoic acid (XLI) is formed. The preferential lactonization of this intermediate to *iso*gladiolic acid (XVII) is no doubt due to steric considerations arising from the presence of the methoxy substituent ortho to one of the carboxyl groups.

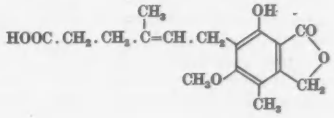


(VII*a*) (XLI) (XVII)

The somewhat involved series of relationships in gladiolic acid chemistry is illustrated in the diagram (p. 652). Gladiolic acid, dihydrogladiolic acid, deoxygladiolic acid and the phenol $C_{10}H_{10}O_3$ make up an interesting series of closely related metabolic products of *P. gladioli*. The obvious structural relationship of the phenol $C_{10}H_{10}O_3$ to mycophenolic acid, and



Phenol $C_{10}H_{10}O_2$



Mycophenolic acid

hence to the series of derivatives of 3:5-dihydroxyphthalic acid discussed by Raistrick (1950), gives the gladiolic acid series of compounds a definite place in the general picture of the metabolic products of the fungi.

EXPERIMENTAL

Micro-analyses are by Weiler and Strauss, Oxford, and by Mr W. Brown. Melting points are corrected. The infrared spectra of solid compounds were obtained using 'Nujol' suspensions in the apparatus described by Grove & Willis (1951).

(1) *Isolation and purification of gladiolic acid*

Penicillium gladioli McCull. & Thom was grown on a simplified Raulin-Thom medium (7·5% glucose) of initial pH 5·0, and the culture filtrate treated with activated charcoal (British Drug Houses Ltd.) followed by elution of the charcoal with ether according to the procedure described by Brian *et al.* (1946) and Brian *et al.* (1948). On removal of the ether by distillation, the crude yellow-brown solid product obtained had, in general, a melting point of 140–155° and further purification was carried out as follows: in a typical experiment, crude gladiolic acid (5 g.) was heated under reflux with ether (500 ml.), a small quantity of amorphous insoluble material was filtered off and discarded and the cooled ethereal solution was extracted with a saturated solution of $NaHCO_3$. Cautious acidification of the yellow bicarbonate extract with dilute HCl brought down a white precipitate which was filtered off and crystallized twice from water (charcoal). The *gladiolic acid* thus obtained was in the form of colourless needles, m.p. 158–160° (2·5 g.), and was sufficiently pure for the preparation of derivatives and for degradative work.

Evaporation of the ether left a small bicarbonate-insoluble fraction in the form of a brown microcrystalline powder, m.p. 155°. Sublimation of this material at 100°/10^{-4} mm. followed by crystallization from ethanol yielded a *phenolic compound*, long colourless needles (12 mg.), m.p. 156–158°, soluble in NaOH solution and giving an intense $FeCl_3$ colour which was blue in both ethanol and aqueous solution. (Found: C, 67·8; H, 6·0%; mol.wt. (Rast), 169. $C_{10}H_{10}O_3$ requires C, 67·4; H, 5·7%; mol. wt., 178.)

(2) *Isolation of a second acidic substance from the culture filtrate*
(with Mrs J. Zealley)

One particular batch of crude product (6 g.) from ether elution of the charcoal extract of the culture filtrate was found to have an abnormally low melting point (120–140°) and was only partly soluble in the normal volume of ether.

The ether-insoluble material (fraction *A*, 395 mg.) could not be purified by sublimation *in vacuo*, but two crystallizations from water gave a colourless crystalline *acid* (47 mg.), m.p. 125–130° (decomp.). The ether-soluble portion yielded gladiolic acid (2·1 g.) by extraction with $NaHCO_3$ in the usual way, but the bulked aqueous mother liquors from the recrystallization of this material gave, on concentration, colourless needles (23 mg.), m.p. 126–128° (decomp.), of an *acid*, identical by mixed melting-point determination with the substance obtained from fraction *A*. It formed colourless needles, m.p. 128–130° (decomp.), from water (found: C, 58·7; H, 5·5%; equiv., 236) and colourless prisms, m.p. 132–135° (decomp.), from ethyl acetate/benzene. (Found: C, 59·0; H, 5·3%; mol.wt. (Rast), 188 (some decomp.); equiv., 230. $C_{11}H_{12}O_5$ requires C, 58·9; H, 5·4%; mol.wt., 224; equiv., 224, monobasic acid.) This acid $C_{11}H_{12}O_5$ gave no coloration with $FeCl_3$ or with ammonia solution, sp.gr. 0·880 (cf. gladiolic acid). It was soluble in ethanol but sparingly soluble in ether. It did not sublime *in vacuo* at 125°. On solution in NaOH, acidification with concentrated HCl and warming, it readily formed a neutral *lactone*, m.p. 170–171°, insoluble in $NaHCO_3$. (Found: C, 64·1; H, 5·0. $C_{11}H_{10}O_4$ requires C, 64·1; H, 4·9%.) This lactone was subsequently shown to be identical by mixed melting-point determinations and comparison of the infrared absorption spectra both with deoxygladiolic acid, $C_{11}H_{10}O_4$, a reduction product of gladiolic acid (see § 8), and with the dihydrogladiolide of Raistrick & Ross (1952). On evaporation of the ethereal solution, the bicarbonate-insoluble fraction proved to be insoluble in NaOH, and gave no coloration with $FeCl_3$. On sublimation at 100°/10^{-4} mm. a colourless sublimate was obtained which yielded, on crystallization from water, two neutral substances the more soluble of which was identical with the lactone $C_{11}H_{10}O_4$ described above. The less soluble substance crystallized from ethanol in needles, m.p. 135–137° (8 mg.), but was not investigated further.

Subsequently, the acid $C_{11}H_{12}O_5$ was shown to be identical with the dihydrogladiolic acid of the London School of Hygiene workers by mixed melting-point determinations and by comparison of the infrared absorption spectra.

(3) *Analysis and properties of gladiolic acid*

For analysis the purified material, m.p. 158–160°, was recrystallized three times from water and formed long colourless needles, m.p. 160°. The analytical specimens

contained no halogen, sulphur or nitrogen and were optically inactive. (Found: C, 59·7, 59·7, 59·4, 59·6; H, 4·6, 4·7, 4·6, 4·4; OCH_3, 13·2%; mol.wt. (Rast), 212; equiv., 228, 207. $C_{11}H_{10}O_5$ requires C, 59·45; H, 4·5; $1OCH_3$, 14·0%; mol.wt., 222.) A Kühn-Roth oxidation gave 4·1% *C*-Me; $C_{11}H_{10}O_5$ requires 6·8% for 1*C*-Me. Gladiolic acid was readily soluble in hot water but only sparingly in the cold. It was very soluble in acetone, dioxan, ethanol and in glacial acetic acid, sparingly soluble in $CHCl_3$ and ether, but only very slightly soluble in carbon tetrachloride, benzene and in light petroleum. It dissolved without charring in cold conc. H_2SO_4; however, it was observed that when conc. H_2SO_4 was added cautiously to a dilute solution of gladiolic acid in technical benzene, an intense red coloration was produced at the benzene/acid interface. Gladiolic acid was soluble in hot dilute mineral acids. It was recovered unchanged in 70% yield after 4 hr. heating under reflux in 2N-H_2SO_4 in an atmosphere of N_2, and no CO_2 or volatile acids were produced. Gladiolic acid dissolved in Na_2CO_3 and in $NaHCO_3$ with liberation of CO_2 and the formation of a faintly yellow solution. The same yellow coloration was also produced on solution in NaOH. The colour changes that occur when gladiolic acid is dissolved in ammonia, sp.gr. 0·880, and the solution is allowed to stand, have already been described (Brian *et al.* 1946). Solid gladiolic acid suspended in air on a spatula over 0·880 ammonia solution is rapidly attacked with the formation of a brown pasty mass; this gives a deep greenish-brown colour in ammonia and acidification of the solution with dilute HCl brings down a heavy blue-green precipitate which may be filtered off and dried. The chemical composition of this material has not been investigated.

Gladiolic acid gave no colour with $FeCl_3$. It did not give iodoform with KI and NaOCl and did not give a red colour with sodium nitroprusside and alkali. The hydroxamic acid test for the carboxyl group (Feigl, 1947) was positive. It did not reduce Fehling's solution or restore the colour to Schiff's reagent; it appeared to reduce ammoniacal silver nitrate in the cold, but observation of this reaction was made difficult by the deep greenish-brown colour produced when gladiolic acid is treated with ammonia. Gladiolic acid reduced $KMnO_4$ on standing in acetone solution at room temperature, but bromine water was not rapidly decolorized even on warming at 100°. Similarly, gladiolic acid did not appear to react with bromine at room temperature either in solution in acetic acid or in suspension in carbon tetrachloride. The nitrochromic acid test for primary and secondary alcoholic —OH groups (Fearon & Mitchell, 1932) was negative. Nevertheless, gladiolic acid contained an —OH group which was readily acetylated (§ 5). It reacted readily with phenylhydrazine and with primary aromatic amines and diamines, but the reactions were complex and simple derivatives were not formed. The *dinitrophenylhydrazone* formed orange needles from glacial acetic acid, m.p. 232° (decomp.), readily soluble in NaOH. In ethanol it gave a red colour with a drop of NaOH. (Found: C, 50·9; H, 3·3; N, 13·8. $C_{17}H_{14}O_8N_4$ requires C, 50·7; H, 3·5; N, 14·0%.)

Formal identification of the alkoxyl substituent. Gladiolic acid (0·1 g.) and hydriodic acid, sp.gr. 1·7 (15 ml.), were maintained at 150° for 1·5 hr. in the conventional type of apparatus and the alkyl iodide produced swept out by a stream of CO_2 and absorbed in redistilled dimethylaniline. A white precipitate formed and was identified as dimethylaniline methiodide, m.p. 204° in a sealed tube, by comparison with a synthetic specimen.

(4) *Reaction with alkali: formation of* iso*gladiolic acid*

(A) *With boiling* 3N-NaOH. Gladiolic acid (0·2 g.) was heated under reflux for 10 min. with 3N-NaOH (10 ml.), the yellow solution cooled and acidified with dilute HCl, and the solid which separated, filtered off and recrystallized from aqueous ethanol. Long colourless needles of iso*gladiolic acid* (0·14 g.), m.p. 234°. (Found: C, 59·3, 59·1; H, 4·4, 4·8; OCH_3, 13·3%; mol.wt. (Rast), 217, 227; equiv., 218, 225. $C_{11}H_{10}O_5$ requires C, 59·45; H, 4·5; OCH_3, 14·0%; mol.wt., 222). A Kühn-Roth estimation gave 3·4% *C*-Me; $C_{11}H_{10}O_5$ requires 6·8% for 1*C*-Me. The infrared spectrum showed two bands in the $\rangle C{=}O$ stretching region at 1758 and 1688 cm.$^{-1}$. *iso*Gladiolic acid was insoluble in benzene and light petroleum and only slightly soluble in ethanol and in $CHCl_3$; it was only slightly soluble in hot water, i.e. much less soluble than gladiolic acid. It dissolved in $NaHCO_3$ solution with liberation of CO_2 and the formation of a colourless solution. *iso*Gladiolic acid (0·104 g.) suspended in water (20 ml.) neutralized 4·96 ml. 0·0928N-NaOH (equiv. 225). After the addition of a further 9·90 ml. NaOH the solution was allowed to stand at room temperature for 18 hr. and then back titrated to the phenolphthalein end point with 0·0826N-H_2SO_4. 5·46 ml. were used, corresponding to an apparent equiv. of 112. *iso*Gladiolic acid did not give the colour reactions with concentrated ammonia solution or with concentrated H_2SO_4 and benzene given by gladiolic acid. It was insoluble in boiling 2N-H_2SO_4. It had no reducing properties, and did not give derivatives either with Brady's reagent or with other reagents for the carbonyl group. It did not contain an acetylatable —OH group and was recovered unchanged (melting point and mixed melting point with starting material) after 4 hr. heating with acetic acid/acetic anhydride, 1·5 hr. heating under reflux with acetic anhydride in pyridine and 6 hr. heating under reflux with acetic anhydride and fused sodium acetate.

The *methyl ester* obtained by heating under reflux with 10% methanolic HCl for 2 hr. formed colourless needles, m.p. 142°. (Found: C, 60·6; H, 5·2. $C_{12}H_{12}O_5$ requires C, 61·0; H, 5·1%.) A mixed melting point with the gladiolic acid methyl ester of m.p. 140° showed a 15° depression. Like *iso*gladiolic acid, methyl *iso*gladiolate did not form a derivative on treatment with Brady's reagent.

(B) *With* 2N-NaOH *at room temperature.* Gladiolic acid (0·1 g.) in 2N-NaOH (5 ml.) was allowed to stand 24 hr. On acidification with conc. HCl, *iso*gladiolic acid (90 mg.), m.p. 232°, was precipitated.

(C) *With* 0·1N-NaOH. Gladiolic acid (0·1 g.) in 0·1N-NaOH (20 ml.) was allowed to stand 30 min. at 25°. On acidification, gladiolic acid, m.p. 156–158°, was recovered unchanged.

(5) *Acetylation of gladiolic acid*

(A) *With acetic anhydride/acetic acid.* (i) Gladiolic acid (0·1 g.) was heated on the water bath with acetic anhydride (1 ml.) and glacial acetic acid (1 ml.) for 1 hr. The reaction was incomplete after only 30 min. heating, but periods in excess of 1 hr. gave a mixture of products (see below) and should not be employed. After cooling and pouring into ice water, the precipitated *neutral monoacetylgladiolic acid* was collected. It crystallized in colourless plates, m.p. 144°, from dilute acetic acid; colourless needles, m.p. 145°, from ethanol. (Found: C, 59·1, 58·8; H, 4·75, 4·6; OCH_3, 12·4;

CH_2CO, 18·8%; mol.wt. (Rast), 256. $C_{13}H_{12}O_6$ requires C, 59·1; H, 4·6; $1OCH_3$, 11·7; $1CH_2CO$, 16·3%; mol.wt., 264.) Acetylgladiolic acid was insoluble in cold NaOH and an aqueous suspension was neutral to the British Drug Houses Ltd. Universal Indicator. It reduced ammoniacal silver solution immediately in the cold, but did not reduce Fehling's solution or restore the colour to Schiff's reagent. It gave no coloration with $FeCl_3$ or with 0·880 ammonia solution. It gave the deep-red colour with concentrated H_2SO_4 and benzene given by gladiolic acid, and contained a very reactive carbonyl group. The *dinitrophenylhydrazone* formed orange needles from glacial acetic acid, m.p. 245–250° (decomp.). (Found: C, 51·3; H, 4·0; N, 12·2. $C_{19}H_{16}O_9N_4$ requires C, 51·3; H, 3·6; N, 12·6%.) The *semicarbazone* crystallized from ethanol in colourless prisms, m.p. 240° (decomp.). (Found: C, 52·5, 52·5; H, 5·0, 5·2; N, 12·4, 12·5. $C_{14}H_{15}O_6N_3$ requires C, 52·3; H, 4·7; N, 13·1%.) Acetylgladiolic acid was converted into *iso*gladiolic acid on hydrolysis with boiling 3N-NaOH. On hydrolysis with boiling 3N-H_2SO_4 acid, gladiolic acid was regenerated and identified by a mixed melting-point determination and by the colour reaction with ammonia.

(ii) When the heating period was extended to 4 hr. using the quantities of gladiolic acid and the acetic anhydride/acetic acid mixture given above, the main product was found to be the *triacetate of gladiolic acid hydrate*, colourless prisms from ethanol, m.p. 131°. (Found: C, 55·4, 55·9; H, 5·1 4·9; OCH_3, 7·6; CH_2CO, 36·5; *C*-Me, 14·8%; mol.wt. (Rast), 323. $C_{17}H_{18}O_9$ requires C, 55·7; H, 4·95; $1OCH_3$, 8·5; $3CH_2CO$, 35·2; 4*C*-Me, 14·8%; mol.wt., 366.) The acetyl determinations were carried out by alkaline hydrolysis followed by acidification, steam distillation and titration of the volatile acid produced. The required *C*-Me content was calculated on the basis of 100% for each CH_2CO group and 60% for the *C*-Me group already detected in gladiolic acid (see § 3) and which gives low values under the standard conditions of the estimation. The low result in the Rast determination of molecular weight is no doubt due to the instability of the compound at the melting point of camphor. The melting point of the triacetyl compound was depressed by acetylgladiolic acid.

The triacetyl compound like acetylgladiolic acid gave no colour with 0·880 ammonia solution and an intense red colour with benzene and concentrated H_2SO_4. It was insoluble in cold NaOH. Unlike acetylgladiolic acid it did not react immediately with carbonyl reagents; but on heating at 100° for 1 hr. with excess semicarbazide acetate, *acetylgladiolic acid semicarbazone*, m.p. 240° (decomp.), was formed in low yield. It did not reduce ammoniacal silver nitrate. On hydrolysis with boiling 3N-NaOH the triacetyl compound was converted into *iso*gladiolic acid.

(iii) The triacetyl compound, m.p. 131°, was also formed though in poor yield (10 mg.) when acetylgladiolic acid (0·2 g.) was heated for 6 hr. on the water bath with the acetic anhydride/acetic acid acetylating mixture (2 ml.).

(B) *With acetic anhydride and concentrated* H_2SO_4. Gladiolic acid (0·1 g.) was suspended in acetic anhydride (1·5 ml.) and a trace of concentrated H_2SO_4 added. The gladiolic acid dissolved at once and after warming for a few minutes the solution was poured on to crushed ice and after working up in the usual way the product was crystallized from ethanol. Colourless prisms, m.p. 131°, identical with the *triacetyl compound* (§ 5 A, ii, above) by a mixed melting-point determination.

(C) *With acetic anhydride and fused sodium acetate.* Gladiolic acid (0·2 g.), acetic anhydride (2 ml.) and fused sodium acetate (0·6 g.) were heated under reflux for 4 hr., and after pouring on to crushed ice, the *acid* product obtained was crystallized from acetone in colourless needles, m.p. 280° (decomp.). (Found: C, 58·6; H, 4·55; OCH_3, 10·2; CH_2CO, 20·7%; equiv., 282. $C_{15}H_{14}O_7$ requires C, 58·8; H, 4·6; $1OCH_3$, 10·1; $1CH_2CO$, 14·1%; equiv. (monobasic acid), 306.) The molecular weight could not be determined in camphor owing to the low solubility of the material. It rapidly reduced $KMnO_4$ in acetone at room temperature and decolorized bromine water on warming. It did not give a derivative with Brady's reagent and ammoniacal silver nitrate was not reduced.

(6) *Esterification of gladiolic acid*

(A) *With methanolic* HCl. Gladiolic acid (0·1 g.) and methanol containing 10% HCl (2 ml.) were heated under reflux for 2 hr. The *methyl ester* separated during the course of the reaction and on cooling was filtered off, washed with water and recrystallized twice from methanol. It formed colourless needles, m.p. 140° (55 mg.). (Found: C, 60·7, 61·1; H, 5·3, 5·1; OCH_3, 23·8; *C*-Me (Kühn-Roth), 7·5%; mol.wt. (Rast), 214. $C_{12}H_{12}O_5$ requires C, 61·0; H, 5·1; $2OCH_3$, 26·2; *C*-Me, 6·4%; mol.wt., 236.) The methyl ester was insoluble in water, cold NaOH and aqueous Na_2CO_3. It gave no coloration with 0·880 ammonia solution or with conc. H_2SO_4. With conc. H_2SO_4 and benzene an intense purple colour was produced at the interface. It did not reduce Fehling's solution, but reduced ammoniacal silver nitrate solution on warming. It contained a reactive carbonyl group. The *dinitrophenylhydrazone*, formed immediately on treatment of an ethanolic solution with Brady's reagent, was a yellow microcrystalline powder, m.p. 245–250° (decomp.). (Found: N, 12·6. $C_{18}H_{16}O_8N_4$ requires N, 13·4%.) The *phenylhydrazone* obtained on warming for a few minutes with phenylhydrazine hydrochloride in aqueous ethanol formed yellowish prisms, m.p. 160°. (Found: N, 8·5. $C_{18}H_{18}O_4N_2$ requires N, 8·6%.)

The *methyl diacetylgladiolate hydrate* was prepared as follows: The ester (0·1 g.) was suspended in acetic anhydride (2 ml.), cooled to 0° and a drop of conc. H_2SO_4 added. The ester rapidly dissolved and the clear solution was allowed to stand for 30 min. at room temperature and then poured into ice water. The product after crystallization from ethanol melted at 106°. (Found: C, 56·9; H, 5·0; OCH_3, 16·2; CH_2CO, 28·2%; mol.wt. (Rast), 312. $C_{16}H_{16}O_8$ requires C, 56·8; H, 5·4; $2OCH_3$, 18·3; $2CH_2CO$, 25·5%; mol.wt., 338.)

The methyl ester was hydrolysed quantitatively by boiling 2N-NaOH to *iso*gladiolic acid, m.p. 232°.

(B) *With ethanolic* H_2SO_4. Gladiolic acid (0·1 g.), absolute ethanol (1 ml.) and a drop of conc. H_2SO_4 were heated under reflux for 30 min., the yellow solution poured into water and the solid product collected. By crystallization from ethanol, colourless needles, m.p. 105°, of the *ethyl ester* were obtained. (Found: C, 61·6; H, 5·7%; mol.wt. (Rast), 244, 262. $C_{13}H_{14}O_5$ requires C, 62·4; H, 5·6%; mol.wt., 250.) The ethyl ester was insoluble in Na_2CO_3, in NaOH and in 0·880 ammonia solutions. It gave no colour with $FeCl_3$. It restored the colour to Schiff's reagent, reduced ammoniacal silver solution in the cold and readily formed a precipitate with Brady's reagent. The *semicarbazone* formed colourless prisms, m.p. 190° (decomp.). (Found: C, 54·9; H, 5·7; N, 13·5. $C_{14}H_{17}O_5N_3$ requires C, 54·7; H, 5·6; N, 13·7%.)

The ethyl ester could be acetylated in the same way as the methyl ester. The ethyl ester (0·1 g.) was suspended in acetic anhydride (1 ml.) and a drop of concentrated H_2SO_4 added. After standing some hours at room temperature the solution was poured on to crushed ice and the *ethyl diacetylgladiolate hydrate* recrystallized twice from ethanol. It formed colourless prisms, m.p. 103°. A mixed melting-point determination with the starting material showed a 20° depression. (Found: C, 57·6; H, 5·4. $C_{17}H_{20}O_8$ requires C, 57·9; H, 5·6%.) *iso*Gladiolic acid was formed when ethyl gladiolate was hydrolysed with hot 3 N-NaOH.

(C) *With diazomethane.* Gladiolic acid (1·0 g.) suspended in ether was treated with an excess of an ethereal solution of diazomethane and allowed to stand 2 days. After removal of the ether and diazomethane the residual gum was distilled at 5×10^{-2} mm. pressure. The distillate which passed over between 180 and 210° partly solidified and the solid portion crystallized from methanol in colourless leaflets, m.p. 105°, but the quantity obtained was too small for further work. The oil was subjected to chromatography on activated alumina using the technique of fractional elution (Shoppee & Reichstein, 1941) and shown to be a mixture of substances; the main fraction contained a small amount of nitrogen (2·7%) and was not further investigated.

(7) *Oxidation of gladiolic acid, isogladiolic acid and related compounds*

(A) *Gladiolic acid.* (i) *With potassium permanganate* ≡ 1 O *atom per molecule gladiolic acid in alkaline solution at room temperature.* 1% $KMnO_4$ solution (10·5 ml.: 1·3 oxygen atoms) was added dropwise from a burette to a well stirred solution of gladiolic acid (0·14 g.) in Na_2CO_3 (15 ml.) at 20°. After filtering off the MnO_2 formed, the solution was acidified with concentrated HCl and evaporated to small bulk. On cooling a white solid crystallized, was collected, and recrystallized from hot water (substance *A*). Colourless needles (0·1 g.), m.p. 219°. (Found: C, 54·8, 55·6; H, 4·2, 4·5; OCH_3, 13·7%; mol.wt. (Rast), 244; equiv., 116. $C_{11}H_{10}O_6$ requires C, 55·4; H, 4·2; $1OCH_3$, 13·0%; mol.wt., 238; equiv. (dibasic acid), 119.) Substance *A* dissolved in $NaHCO_3$ with liberation of CO_2 and the formation of a colourless solution. It did not give the colour reactions with ammonia, and with benzene and H_2SO_4 given by gladiolic acid. No red coloration was produced on fusing with a crystal of phenol and a trace of conc. H_2SO_4, diluting with water and adding NaOH. The fluorescein test was also negative. It was readily acylated under the conditions used for the acetylation of gladiolic acid (§ 5).

Substance *A* (50 mg.) heated 2 hr. on the water bath with acetic anhydride (1 ml.) and acetic acid (1 ml.) yielded after working up in the usual way, the *monoacetate*, colourless needles from dilute acetic acid, m.p. 199°. (Found: C, 55·8; H, 4·4%; equiv., 259. $C_{13}H_{12}O_7$ requires C, 55·7; H, 4·3%; mol.wt., 280.)

Substance *A* did not give an immediate precipitate on warming with Brady's reagent, but carbonyl derivatives were formed under somewhat more drastic conditions. Substance *A* (50 mg.) dissolved in boiling 2 N-HCl was added to 2:4-dinitrophenylhydrazine (50 mg.) in 2 N-HCl and the whole heated under reflux for 5 min. and then at 100° for 30 min. The *dinitrophenylhydrazone* was obtained as a yellow microcrystalline powder, m.p. 245–250° decomp., giving a deep-red solution in NaOH. (Found: C, 48·4; H, 3·1; N, 13·7. $C_{17}H_{14}O_9N_4$ requires C, 48·8; H, 3·4; N, 13·4%.)

Substance *A* (20 mg.) and phenylhydrazine hydrochloride (30 mg.) were heated in aqueous solution at 100° for 2 hr. On cooling, the *anhydrophenylhydrazone* separated, m.p. 190° decomp., soluble in Na_2CO_3. (Found: C, 65·0; H, 4·7; N, 9·5%; equiv., 282. $C_{17}H_{14}O_4N_2$ requires C, 65·8; H, 4·55; N, 9·0%; equiv., 310, monobasic acid.)

The *semicarbazone* formed colourless prisms, m.p. 192° decomp. (Found: C, 48·5; H, 4·6; N, 13·2%; equiv., 154. $C_{12}H_{13}O_6N_3$ requires C, 48·8; H, 4·4; N, 14·2%; equiv., 147·5, dibasic acid.)

Substance *A* was recovered unchanged after boiling for 10 min. with 3 N-NaOH followed by cooling and acidification. It was similarly unaffected by standing overnight with 40% aqueous KOH.

(ii) *Excess alkaline potassium permanganate at room temperature.* Gladiolic acid (1·0 g.), Na_2CO_3 (20 ml.) and 5% $KMnO_4$ (40 ml.) were stirred together for 12 hr.; after removal of the MnO_2, the filtrate was acidified, the excess permanganate removed with SO_2, and the solution concentrated to small bulk. In one experiment a small amount (70 mg.) of a colourless acid, m.p. 218°, separated and was found to be substance *A*, but in general inorganic material was the first to separate and the solution was evaporated to dryness and the solid product extracted with ether in a Soxhlet apparatus. Removal of the ether left a gummy solid which crystallized from ethyl acetate in colourless prisms, substance *B* (0·12 g.), m.p. 162°. A much better yield of substance *B* (0·85 g. from 1·0 g. gladiolic acid) was obtained when the solution, after acidification and destruction of the excess permanganate, was continuously extracted with ethyl acetate. (Found: C, 52·1; H, 3·9; OCH_3, 12·5%; mol.wt. (Rast), 222; equiv., 85. $C_{11}H_{10}O_7$ requires C, 52·0; H, 4·0; $1OCH_3$, 12·2%; mol.wt., 254; equiv., 85, tribasic acid.) Substance *B* was very soluble in water. It gave no colour with $FeCl_3$, but gave strongly positive colour reactions for an *o*-phthalic acid (fluorescein and phenolphthalein tests). The melting point of 162° recorded above was unaffected by several cycles of resetting and remelting and was unchanged even after the melt had been maintained at 200° for 10 min. The infrared spectrum showed a strong band at 927 cm.$^{-1}$ absent in the spectra of substances *C* and *D* (see below). Substance *B* (0·12 g.) in methanol, treated with an excess of ethereal diazomethane and allowed to stand 24 hr., formed the *trimethyl ester* (90 mg.), colourless prisms from methanol m.p. 98°. (Found: C, 56·9; H, 5·85; OCH_3, 38·3. $C_{14}H_{16}O_7$ requires C, 56·75; H, 5·45; $4OCH_3$, 41·9%.)

(iii) *Excess boiling potassium permanganate in sodium carbonate.* (*a*) Gladiolic acid (0·2 g.) was dissolved in Na_2CO_3 (10 ml.) and 5% $KMnO_4$ added dropwise to the boiling solution. At the end of 30 min. when 10·7 ml. had been added a permanent pink colour remained 5 min. after the addition of four drops of permanganate solution. After removal of the MnO_2 and acidification with conc. HCl, the solution was evaporated cautiously to dryness and the residue extracted with ether. The colourless solid obtained, substance *C*, m.p. 160–165° decomp., was very soluble in water and in ether. (Found: C, 49·7, 50·0; H, 3·7, 4·0; OCH_3, 14·6%; mol.wt., 232; equiv., 82. $C_{10}H_8O_7$ requires C, 50·0; H, 3·4; OCH_3, 12·9%; mol.wt., 240; equiv., 80, tribasic acid.) Substance *C* gave positive results in the usual tests for an *o*-phthalic acid. The melting point was not depressed by admixture with substance *B*, but the infrared spectrum showed bands at 1123 and 1143 cm.$^{-1}$ which were absent in the spectra both of substances *B* and *D* (see below).

(*b*) Gladiolic acid (1·0 g.) was dissolved in Na_2CO_3 (20 ml.) and oxidized with 5% $KMnO_4$. The reaction was started at room temperature and when uptake of permanganate ceased, was continued by heating under reflux for 4 hr. In all, 53·0 ml. of permanganate solution were used, corresponding to approx. 5 O atoms per molecule of gladiolic acid. After removal of the MnO_2 and acidification, the solution was evaporated to small bulk and extracted with 5 × 10 ml. portions of ethyl acetate. Evaporation of the solvent left a colourless solid (0·2 g.), m.p. 171–181° decomp., with gas evolution (fraction 1). The aqueous solution on standing deposited a crop of colourless crystals (0·45 g., fraction 2), insoluble in ethyl acetate, but readily soluble in water. These on heating lost water at 120° and then remelted with decomposition at 280°. The crystals did not sublime at 210° and 10^{-4} mm. Hg pressure, and a white residue remained after ignition on a porcelain tile.

Fraction 1 was sublimed at 130°/10^{-4} mm. The sublimate (30 mg.), m.p. 152–164° decomp. after recrystallization from ethyl acetate, was identical with substance *C* by comparison of the infrared absorption spectra. The residue was crystallized from ethyl acetate and obtained as a microcrystalline powder, substance *D*, m.p. 175–180° decomp., with gas evolution, resetting at 220° and remelting at 259° with further decomp. (Found on a specimen dried *in vacuo* over P_2O_5 at room temperature: C, 43·8; H, 3·7%; equiv., 78·1. $C_{11}H_8O_9.H_2O$ requires C, 43·5; H, 3·3%; equiv., 75·5 (tetrabasic acid). Found on a specimen dried at 100° *in vacuo* over P_2O_5: C, 46·3, 47·3; H, 2·8, 2·95; OCH_3, 9·9, 11·4%; mol.wt. (Rast), 304; equiv., 74·5. $C_{11}H_8O_9$ requires C, 46·5; H, 2·8; OCH_3, 10·9%; mol.wt., 284; equiv., 71, tetrabasic acid.)

Substance *D* crystallized from a small volume of dilute HCl in colourless needles, m.p. 190°, with gas evolution, resetting at 210° and remelting at 259° decomp. (Found on a specimen dried 3 hr. *in vacuo* over P_2O_5 at room temperature: C, 43·5; H, 3·3. $C_{11}H_8O_9.H_2O$ requires C, 43·5; H, 3·3%. Found on a specimen dried 6 hr. *in vacuo* over P_2O_5 at 100°: C, 46·7; H, 2·95. $C_{11}H_8O_9$ requires C, 46·5; H, 2·8%.)

Substance *D* did not depress the melting point of substance *B* or substance *C*. The infrared spectrum showed a strong band at 1165 $cm.^{-1}$ and weak bands at 865 and 940 $cm.^{-1}$, which were absent in the spectra of substances *B* and *C*. It was very soluble in water but only slightly soluble in ether. It showed positive reactions in the usual tests for an *o*-phthalic acid. It gave no coloration with $FeCl_3$.

Fraction 2 was boiled for a few minutes with conc. HCl (5 ml.), adding a little water to dissolve the KCl formed. On cooling, colourless needles of substance *D* (0·15 g.), m.p. 180°, remelting at 256° (decomp.) were obtained. Extraction of the acid solution with ethyl acetate yielded a further small quantity (20 mg.) of the same product.

Substance *D* (0·5 g.) in methanol treated with an excess of ethereal diazomethane and allowed to stand 1 week formed the *tetramethyl ester*, a colourless oil (0·4 g.), b.p. 180–182°/10^{-2} mm. (Found: C, 53·1; H, 4·8; OCH_3, 41·5. $C_{15}H_{16}O_9$ requires C, 52·9; H, 4·7; 5OCH_3, 45·5%.)

(iv) *With excess boiling potassium permanganate in potassium hydroxide.* Gladiolic acid (2·0 g.) in boiling N-KOH (25 ml.) consumed 135 ml. 5% $KMnO_4$, and 0·95 g. crude substance *D*, m.p. 175–180° decomp., was obtained by ethyl acetate extraction of the concentrated aqueous filtrate. Sublimation of this crude material at 150°/10^{-3} mm. did not yield any substance *C*.

(v) *Attempted oxidation with periodic acid.* The method adopted was essentially that of Clutterbuck & Reuter (1935). In a preliminary experiment gladiolic acid (0·1322 g.) was dissolved in water (100 ml.), a standard approx. 0·1 N solution of HIO_4 (100 ml.) added and the mixture allowed to stand for 48 hr. at room temperature. At the end of this time the solution was titrated, after the addition of acid and excess KI, against standard $Na_2S_2O_3$ solution. The difference between this titre and a blank titration gave the amount of periodate consumed. In this experiment it corresponded to only 32% of the theoretical for the oxidation of 1 mol. of gladiolic acid by 1 mol. of periodic acid.

In a second experiment using 0·1277 g. gladiolic acid, the solution in 0·1 N-HIO_4 (100 ml.) and 3 N-HCl (10 ml.) was made up to 250 ml. and allowed to stand with occasional shaking at 30°. 10 ml. Portions were withdrawn at intervals and titrated in the usual way:

Time (days)	1	3	6	9	12	21
Percentage 1 mol. periodic acid consumed per mol. gladiolic acid	17	26	34	55	55	68

At the end of 3 weeks, concentration of the residual solution yielded 0·055 g. (42%) pure gladiolic acid, colourless needles, m.p. 156°.

Repetition of this experiment on a somewhat larger scale (0·5 g. gladiolic acid) gave very similar results.

(B) iso*Gladiolic acid. Excess potassium permanganate in alkali at* 100°. *iso*Gladiolic acid (1·1 g.) was dissolved in N-KOH (20 ml.), the solution heated to boiling with vigorous mechanical stirring and oxidized in the usual way with 5% $KMnO_4$ solution added dropwise from a burette. After the addition of 50 ml. the rate of consumption of the permanganate was reduced considerably; a further 20 ml. were then added and the boiling continued 2 hr. more. At the end of this period the excess permanganate was destroyed by the addition of methanol and the mixture cooled and the MnO_2 filtered off and washed with N-KOH solution. After acidification of the bulked filtrate and washings, the solution was concentrated and continuously extracted with ethyl acetate for some hours. Concentration of the ethyl acetate gave 0·57 g. of an *acid*, m.p. 175–180° decomp., with gas evolution, resetting at 220° and remelting at 256° (decomp.). This behaviour on heating was unaltered by admixture with substance *D* from gladiolic acid.

(C) *Acetylgladiolic acid.* Acetylgladiolic acid (0·1 g.) was dissolved in acetone (2 ml.) and solid $KMnO_4$ (0·09 g.) added. After standing 1 hr. at room temperature, water (2ml.) and saturated Na_2CO_3 (1 ml.) were added and the MnO_2 removed by filtration. The filtrate was acidified with concentrated HCl and after standing overnight at 0° the colourless crystals were filtered off and recrystallized from 50% aqueous ethanol. Colourless prisms, m.p. 213° (20 mg.), soluble in cold Na_2CO_3 solution with liberation of CO_2. (Found: C, 55·8, 55·5; H, 4·5, 4·2%; mol.wt. (Rast), 272; equiv., 263. $C_{13}H_{12}O_7$ requires C, 55·7; H, 4·3%; mol.wt., 280.) On the addition of a single crystal of this *acid* to a melt maintained at 205° of the needles of the acetate of substance *A*, m.p. 199°, the whole solidified immediately and remelted at 213°. In a mixed melting-point determination the mixture melted at the higher temperature (213°).

(D) *Substance A.* (i) *With acidified potassium permanganate at* 100°. Substance *A* (0·1 g.) was dissolved by warming in 5% H_2SO_4 (5 ml.) and 1% $KMnO_4$ added to the boiling

solution. On cooling, unchanged substance *A* separated, identified by melting point and mixed melting point.

(ii) *With potassium permanganate in alkaline medium at room temperature.* Substance *A* (0·1 g.), Na_2CO_3 (2 ml.) and 1% $KMnO_4$ solution (5 ml.) were stirred at 20°. After 12 hr. all the permanganate had been used up and a further 5 ml. were added and stirring continued 12 hr. more. After removal of the MnO_2, acidification of the filtrate and extraction with ethyl acetate, a colourless solid (70 mg.), m.p. 158–162°, was obtained, which gave no depression in a mixed melting-point determination with substance *B*. (Found: C, 52·1; H, 4·1%; equiv., 92. $C_{11}H_{10}O_7$ requires C, 52·0; H, 4·0%; equiv., 85, tribasic acid.)

(E) *Deoxygladiolic acid.* Deoxygladiolic acid (15 mg.) was dissolved in N-H_2SO_4 (30 ml.) at 70° and 1% $KMnO_4$ solution (2 ml.) added. After 10 min. the excess permanganate was removed with SO_2 and on cooling overnight at 0° the solution deposited colourless needles, m.p. 230°, identical with *iso*gladiolic acid by mixed melting point and comparison of the infrared spectra.

(8) *Reduction of gladiolic acid*

(A) *With iron powder and acetic acid.* Gladiolic acid (0·5 g.), activated iron powder (1·0 g.) and 50% acetic acid (10 ml.) were heated on the steam bath for 2 hr. The dark-coloured solution was diluted with an equal volume of water and extracted with ether. Removal of the ether and residual acetic acid left a yellow pasty material (0·15 g.) which was taken up in ether and extracted with $NaHCO_3$. The neutral fraction crystallized from ethanol in stout colourless needles of *deoxygladiolic acid*, m.p. 173° (60 mg.). The melt partly resolidified on maintaining at 180° for a few minutes. (Found: C, 63·8; H, 5·05; OCH_3, 14·2%; mol.wt. (Rast), 215. $C_{11}H_{10}O_4$ requires C, 64·1; H, 4·9; OCH_3, 15·0%; mol.wt., 206.) The infrared spectrum showed bands at 1758 and 1695 cm.$^{-1}$ due to >C=O groups. Deoxygladiolic acid was insoluble in cold NaOH, but readily dissolved on warming. 12·0 mg. deoxygladiolic acid were dissolved in 5·06 ml. 0·128 N-NaOH by warming on the water bath. Back titration with 0·128 N-HCl to the phenolphthalein end point required 4·62 ml., whence the apparent equivalent weight is 213. It gave a *dinitrophenylhydrazone* decomp. above 320° on treatment with Brady's reagent. (Found: N, 14·8. $C_{17}H_{14}O_7N_4$ requires N, 14·5%.) Deoxygladiolic acid was identical by mixed melting-point determination and comparison of the infrared spectra both with the lactone obtained from crude gladiolic acid and from dihydrogladiolic acid and with the dihydrogladiolide of the London School of Hygiene workers (§ 2). The bicarbonate-soluble fraction on acidification and extraction with ether yielded only a trace of acidic material, m.p. 125–135°.

(B) *By Clemmensen's method.* Granulated zinc (10 g.), $HgCl_2$ (1 g.), concentrated HCl (0·5 ml.) and water (10 ml.) were shaken together for 5 min. after which the aqueous solution was decanted and the zinc covered with water (7·5 ml.) and conc. HCl (10 ml.). Gladiolic acid (1·0 g.) was added and the mixture heated under reflux for 5 hr. The hot solution was decanted from tarry by-products and on cooling at 0° deposited a *neutral lactone*, a colourless, crystalline solid which was recrystallized from water (0·25 g.), m.p. 113°. (Found: C, 68·4, 68·9; H, 6·5, 6·5%; mol.wt. (Rast), 212; equiv. (by lactone titration), 188. $C_{11}H_{12}O_3$ requires C, 68·7; H, 6·3%; mol. wt., 192.) The lactone >C=O group absorbed at 1745 cm.$^{-1}$ in the infrared. This reduction product was insoluble in cold NaOH, but was readily soluble on warming and was precipitated unchanged therefrom by dilute HCl. It showed no carbonyl reactivity. The tarry by-products were treated with ethanol and the insoluble material crystallized from acetone in colourless needles, m.p. 282–286° decomp., which could be sublimed at 250°/10^{-4} mm. without change of melting point (10 mg.). (Found: C, 69·1; H, 5·5. $C_{22}H_{20}O_6$ requires C, 69·6; H, 5·3%.) This substance was also neutral and non-ketonic.

(9) *Demethylation of degradation products*

(A) *Substance D.* Substance *D* (0·1 g.) and HBr, sp.gr. 1·49 (4 ml.), were heated under reflux for 30 min. The *hydroxybenzenetetracarboxylic acid* separating on cooling was dried *in vacuo* over H_2SO_4 and crystallized from a small volume of water in colourless prisms, m.p. 302° decomp. It gave a red coloration with $FeCl_3$. (Found on a specimen dried 6 hr. *in vacuo* at 100° over P_2O_5: C, 41·7, 41·7; H, 2·9, 2·9%; equiv., 75, 73. $C_{10}H_6O_{10}$ ($C_{10}H_4O_9 . H_2O$) requires C, 41·7; H, 2·8%; equiv., 72, tetrabasic acid.) It coupled very slowly with diazotized amines, but both the Millon and Gibbs tests were negative. On methylation with diazomethane in methanol the tetramethyl ester of substance *D* was formed and identified from its infrared spectrum.

(B) iso*Gladiolic acid.* (*a*) *iso*Gladiolic acid (0·2 g.) and HBr (7 ml.) were heated under reflux for 40 min. On cooling *norisogladiolic acid* separated and crystallized from aqueous ethanol in prisms, m.p. 254–256° decomp. (Found: C, 57·75; H, 3·9%; equiv., 196. $C_{10}H_8O_5$ requires C, 57·7; H, 3·9%; mol.wt., 208.) In the >C=O region the infrared spectrum showed a broad band with maximum at 1695 cm.$^{-1}$ and a shoulder at 1727 cm.$^{-1}$. Nor*iso*gladiolic acid gave a beautiful reddish-purple colour with ferric chloride. It coupled immediately with diazotized *p*-nitraniline, but only very slowly with diazotized aniline. It gave a negative Millon reaction, but with the Gibbs reagent a faint blue colour developed slowly. (*b*) *iso*Gladiolic acid (0·15 g.) and HI, sp.gr. 1·7 (6 ml.), were heated under reflux for 3 hr. in an atmosphere of N_2, cooled, diluted with water and extracted with ether. The ethereal extract was washed successively with water, $Na_2S_2O_3$ and water, dried, and the ether removed. Nor*iso*gladiolic acid, m.p. 250–255° decomp., was obtained from the tarry residue by crystallization from ethanol (charcoal).

(C) *Gladiolic acid.* When gladiolic acid (0·1 g.) was heated under reflux with HBr (2 ml.) as described under *iso*gladiolic acid above, the tarry product, after purification by sublimation *in vacuo*, was identified as *norisogladiolic acid* by a mixed melting-point determination. (Found: C, 57·6; H, 4·1. $C_{10}H_8O_5$ requires C, 57·7; H, 3·9%.)

(D) *Clemmensen reduction product* $C_{11}H_{12}O_3$. The compound (60 mg.) was heated under reflux with HBr (2 ml.) for 20 min. when a solid product began to separate. After filtration and drying over KOH, it crystallized from ethanol in needles, m.p. 158°. (Found: C, 67·2; H, 5·7%; equiv. (by lactone titration), 175. $C_{10}H_{10}O_3$ requires C, 67·4; H, 5·7%; mol.wt., 178.) The infrared spectrum showed bands at 3378 and 1745 cm.$^{-1}$ due to —OH and >C=O groups respectively. It gave an intense blue colour with $FeCl_3$ in

ethanol and in aqueous solution. It did not couple with diazotized *p*-nitraniline and showed negative Millon and Gibbs reactions. It was identical by mixed melting point and by comparison of the infrared absorption spectra with the *phenol* isolated from crude gladiolic acid (§ 1).

(E) *Deoxygladiolic acid.* Deoxygladiolic acid (30 mg.) was cautiously dissolved in HBr (2 ml.) by warming on the steam bath and then heated under reflux for a few minutes. Heating was stopped as soon as red amorphous material began to appear and the solution cooled. The amorphous precipitate was filtered off, the filtrate diluted with water (6 ml.) and extracted with ether, and the ethereal layer extracted with cold 0·1 N-NaOH. The yellow alkaline layer was acidified with concentrated HCl and extracted with ether. Removal of the ether left a solid substance purified by sublimation *in vacuo* at 150°/10^{-3} mm. followed by crystallization from ethanol affording *nordeoxygladiolic acid* as colourless plates, m.p. 205–208° decomp. (Found: C, 62·3; H, 4·7. $C_{10}H_9O_4$ requires C, 62·5; H, 4·2%.) It readily sublimed at 200°, gave a reddish-violet $FeCl_3$ colour and formed a precipitate with Brady's reagent. In the infrared spectrum the $\rangle C{=}O$ groups absorbed at 1733 and 1688 cm.$^{-1}$.

(10) *Decarboxylation of* isogladiolic *acid and gladiolic acid*

(A) iso*Gladiolic acid.* *iso*Gladiolic acid (0·5 g.) in redistilled quinoline (7 g.) was heated in a stream of CO_2-free N_2 with a copper chromite catalyst (0·2 g.). CO_2 was rapidly evolved at a bath temperature of 220° and was swept out and absorbed in a series of wash bottles containing standard baryta. The CO_2 evolved after 2·5 hr. corresponded to 87% of 1 mol. CO_2/mol. of *iso*gladiolic acid and the reaction mixture was cooled, the catalyst filtered off and the filtrate poured into excess 2 N-HCl (25 ml.). The solution was extracted with ether and the tarry ethereal extract sublimed at 200°/10^{-3} mm. The orange-coloured sublimate crystallized from ethanol in colourless needles, m.p. 244° (27 mg.). (Found: C, 65·7, 66·2; H, 4·7, 5·1; OCH_3, nil; mol.wt. (Rast), 186. $C_9H_6O_3$ requires C, 65·8; H, 4·9%; mol.wt., 164.) It was soluble in cold NaOH, but insoluble in $NaHCO_3$ and an aqueous suspension was neutral to the Universal indicator. It gave no colour with $FeCl_3$. The Millon reaction was negative. The infrared spectrum showed a band at 1738 cm.$^{-1}$ attributed to a phthalide ring $\rangle C{=}O$ group.

(B) *Gladiolic acid.* Under precisely the same conditions 95% of 1 mol. of CO_2 was evolved from gladiolic acid in 30 min. at 220°, but the tarry product obtained by ether extraction proved intractable.

(11) *Synthesis of* 4-*methoxybenzene*-1:2:3:5-*tetracarboxylic acid* (with Mrs J. Zealley)

Mesityl methyl ether. Nitromesitylene was converted in 86% yield into mesidine by hydrogenation at 25° in the presence of a Raney nickel catalyst. Diazotization of mesidine and replacement of $—NH_2$ by —OH (Porter & Thurber, 1921) gave mesitol which was methylated with dimethyl sulphate and caustic soda in the usual way. The mesityl methyl ether had b.p. 205–208°.

Acylation of mesityl methyl ether. (*a*) Powdered anhydrous $AlCl_3$ (13·5 g.) was added with stirring to CS_2 (20 ml.) in a flask fitted with a mechanical stirrer, dropping funnel and reflux condenser and immersed in an ice bath, and a mixture of mesityl methyl ether (10 g.) and acetyl chloride (7·5 g.) added at such a rate that the temperature did not exceed 5°. The solution was then stirred for 2 hr. at 20° and decomposed with ice and HCl in the usual way. The oily layer was extracted with ether and after removal of the ether and CS_2 the residue was taken up again in ether and extracted with NaOH. Acidification of the aqueous alkaline layer followed by extraction with ether yielded only a trace of phenolic material. The neutral oil from the ether layer was distilled and fractions, b.p. 220–240° (2·0 g.) and 250–275° (6·4 g.), collected. The forerun, b.p. > 220°, consisted of unchanged mesityl methyl ether. The yield of oil, b.p. 250–275°, was not improved by carrying out the reaction at 45° after the addition of mesityl methyl ether and acetyl chloride to the $AlCl_3$ at 5°.

(*b*) Mesityl methyl ether (4·2 g.) and powdered anhydrous $AlCl_3$ (12 g.) in CS_2 (20 ml.) were stirred together and acetic anhydride (3·0 g.) added at room temperature. The temperature of the reaction mixture rose to 35°, and after stirring for 2 hr. the product was worked up as before. Fractions, b.p. 200–240° (0·82 g.) and 255–275° (2·65 g.), were collected.

The infrared absorption spectra of the high boiling fractions from both experiments showed two bands in the $\rangle C{=}O$ stretching region at 1760 and 1700 cm.$^{-1}$ respectively. The former frequency is characteristic of phenyl ester $\rangle C{=}O$ and the latter of aryl alkyl ketone $\rangle C{=}O$ (Grove & Willis, 1951).

The oil, b.p. 250–275° (4·4 g.) and 3 N-NaOH (40 ml.), were heated under reflux for 1 hr. and the oily layer remaining extracted with ether. Removal of the ether and distillation of the residual oil gave 3-methoxy-2:4:6-trimethylacetophenone, b.p. 268–270° (3·0 g.). (Found: C, 74·8; H, 8·5; OCH_3, 14·9. $C_{12}H_{16}O_2$ requires C, 75·0; H, 8·4; OCH_3, 16·2%.)

Acidification of the aqueous alkaline layer and repeated recrystallization from petroleum ether, b.p. 40–60°, of the solid obtained gave 3-hydroxy-2:4:6-trimethylacetophenone, m.p. 81–82° (0·4 g.). (Found: C, 74·2; H, 8·1. Calc. for $C_{11}H_{14}O_2$: C, 74·1; H, 7·9%.) Auwers & Mauss (1928) give m.p. 81° for 3-hydroxy-2:4:6-trimethylacetophenone. Alkaline hydrolysis of the fraction, b.p. 200–240°, in a similar manner gave mesityl methyl ether, and mesitol, melting point and mixed melting point with an authentic sample, 71°.

4-*Methoxybenzene*-1:2:3:5-*tetracarboxylic acid.* 3-Methoxy-2:4:6-trimethylacetophenone was dispersed by stirring in saturated Na_2CO_3 (20 ml.) and 5% $KMnO_4$ added slowly first to the warm and then to the boiling solution. The reaction was continued by heating under reflux for 4 hr. by which time 300 ml. permanganate solution had been added. The excess of $KMnO_4$ was destroyed by the addition of methanol, the MnO_2 filtered off and washed with Na_2CO_3 and the bulked filtrate and washings acidified to congo red with concentration HCl and evaporated to 100 ml. Continuous extraction of this solution with ether furnished a fluffy amorphous solid (1·9 g.) which was markedly hygroscopic. It was dissolved in 2 N-H_2SO_4 (20 ml.) and 5% $KMnO_4$ added to the warm solution. CO_2 was evolved and when uptake of permanganate ceased, the solution was cooled, made strongly acid with concentrated HCl and continuously extracted with ethyl acetate. Removal of the solvent left

a solid product (1·12 g.), m.p. 175–180° decomp., resetting at 220° and remelting at 256° (decomp.), and identical by mixed melting point and comparison of the infrared spectrum with substance *D*, the methoxybenzenetetracarboxylic acid from gladiolic acid. For analysis, a portion of the material was crystallized from ethyl acetate and dried *in vacuo* over P_2O_5 at 100° for 12 hr. (Found: C, 46·8; H, 3·1%; equiv., 72. $C_{11}H_8O_9$ requires C, 46·5; H, 2·8%; equiv., 71, tetrabasic acid.)

4-Hydroxybenzene-1:2:3:5-tetracarboxylic acid. 4-Methoxybenzene-1:2:3:5-tetracarboxylic acid (100 mg.) was heated under reflux with hydrobromic acid for 30 min. On cooling 4-hydroxybenzene-1:2:3:5-tetracarboxylic acid separated and formed colourless prisms, m.p. 302°, decomp. from 2N-HCl. (Found: C, 42·0; H, 3·0; equiv., 72. $C_{10}H_6O_9 \cdot H_2O$ requires C, 41·7; H, 2·8%; equiv., 72, tetrabasic acid.) It was identical with the hydroxybenzenetetracarboxylic acid from gladiolic acid by mixed melting-point determinations and comparison of the infrared spectra.

SUMMARY

1. Chemical work is described showing that gladiolic acid $C_{11}H_{10}O_5$, a weakly antibacterial but strongly antifungal metabolic product of *Penicillium gladioli*, may be represented by the tautomeric structural formulae 4-methoxy-5-methyl-*o*-phthalaldehyde-3-carboxylic acid (in the keto form) and 4-formyl-3-hydroxy-7-methoxy-6-methylphthalide (in the lactol form).

2. The structures of a number of derivatives and degradation products of gladiolic acid have also been established and the synthesis of 4-methoxybenzene-1:2:3:5-tetracarboxylic acid is reported.

3. Three impurities, an acid $C_{11}H_{12}O_5$, a lactone $C_{11}H_{10}O_4$ and a phenolic compound $C_{10}H_{10}O_3$, have been isolated from the crude gladiolic acid and their respective structural relationships to gladiolic acid established.

4. The structural relationship of the phenol $C_{10}H_{10}O_3$ to mycophenolic acid gives the gladiolic acid series of compounds a definite place in the general picture of the metabolic products of the fungi.

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The Behaviour of Proteins at the Liquid-Liquid Interface

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An interesting recent development in the study of unimolecular films has been the employment of such films in the determination of the molecular weights of proteins. Guastalla (1939, 1943), working with his sensitive floating-thread surface balance, made the surprising discovery that unimolecular films of proteins at the surface of water behaved in the manner of non-ideal two-dimensional gases at low surface pressures. He was consequently able, by application of the gas laws, to estimate the molecular weights of a number of proteins in the surface phase.

The equation of state for an ideal two-dimensional gas may be written:

$$FA = nRT, \qquad (1)$$

where F represents the surface pressure in dynes/cm., A the area of the gas in sq.cm., n the number of moles of gas, T the absolute temperature and R the two-dimensional gas constant with a value of $8{\cdot}31 \times 10^7$ ergs/mole/degree (cf. Adam, 1941).

For non-ideal surface gases, Volmer & Mahnert (1925) suggested a formal analogy with the van der Waals equation for real gases:

$$(F + a/A^2)(A - b) = RT, \qquad (2)$$

where a is a constant determined by the forces of attraction between the molecules in the surface and b is a constant proportional to the area occupied by the molecules in the surface.

The plots of FA against F obtained by Rideal & Schofield (1925, 1926) for adsorbed films of the lower fatty acids, and by Adam & Jessop (1926) for insoluble films of certain long-chain dibasic esters at the water surface, have the characteristics required by such an equation of state, namely a minimum related to the a/A^2 term and an intercept on the FA-axis approximating to the theoretical value of RT. There appears to be little doubt that an equation of the van der Waals type will describe the behaviour of a gaseous surface film, at least in the first approximation.

Guastalla (1939, 1943), for his protein films, plotted the quantity C/F against C, where C represented the surface concentration in mg. protein/sq.m. The curves thus obtained showed a linear portion at low concentrations which could be extrapolated to $C = 0$. The intercept on the C/F axis gave a value for $1/nRT$ from which the molecular weight of the protein could be calculated. The values found for gliadin (27,000) and egg albumin (40,000) agreed well with the accepted values from osmotic pressure measurements in bulk phase. On the substrate employed (0·01 N-HCl) haemoglobin apparently underwent dissociation into fragments of mean molecular weight 12,000.

Bull (1945, 1946, 1947, 1950) and Dieu & Bull (1949) have reported an extensive series of experiments on films of proteins at the air-water interface, mainly carried out with a surface balance of the Wilhelmy type. They find that all such films, with the exception of zein, give FA-F plots in which no minima occur. In many cases the relation is quite rectilinear, suggesting an absence of appreciable interaction between the protein molecules, and extrapolation to $F = 0$ may be performed with ease. Bull gives the equation for his straight-line graphs in the empirical form:

$$FA = \alpha F + \beta, \qquad (3)$$

where α represents the slope of the line and β the intercept on the FA-axis. With the assumption of the validity of the ideal gas laws at zero surface pressure, β will then give the value of nRT and the molecular weight of the molecules constituting the film may be calculated. When F is expressed in dynes/cm. and A in sq.m./mg. protein, it is clear that at 25°:

$$\beta = 8{\cdot}31 \times 298 \times 10^7 \times n \text{ ergs},$$

whence the molecular weight

$$M = \frac{8{\cdot}31 \times 298}{\beta} = \frac{2460}{\beta}.$$

The slope α of the line will give the area in sq.m./mg. occupied by the protein molecules in the uncompressed state.

In the most recent of his papers, Bull (1950) gives a thermodynamic derivation of Eqn. 3 in which the constants prove to have the meaning previously attributed to them. This derivation tacitly assumes an absence of interaction between the molecules constituting the surface layer, an approximation that appears to be justified by the experimental results.

On the basis of these considerations, Bull has found molecular weights for egg albumin, bovine

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serum albumin, insulin, rabbit γ-globulin, pepsin, trypsin, chymotrypsinogen and trypsinogen, all spread on ammonium sulphate solutions, corresponding within the limits of experimental error to values previously established by osmotic pressure measurements and with the ultracentrifuge. Zein, to which Bull (1947) only alludes in passing, is peculiar in that the FA-F curve shows a minimum and the molecular weight (20,100) shows dissociation to have taken place on the surface. β-Lactoglobulin shows half the bulk-phase molecular weight (17,100) on ammonium sulphate. When, however, copper ions are present in the substrate, a value showing no dissociation (34,300) is found.

The data at present available concerning the behaviour of protein films at the interface between two liquids (Danielli, 1938; Askew & Danielli, 1940; Alexander & Teorell, 1939; Cheesman, 1946*a*; Cumper & Alexander, 1950) indicate that at areas above 1 sq.m./mg. a film of a given protein exerts under such conditions considerably higher pressures than does a film of the same protein at the air-water interface. It would seem that no investigation has been made into the state of the interfacial protein films at large areas, although Cumper & Alexander (1950) have referred to them as being of the vapour-expanded type. The findings of Bull, however, demonstrate clearly the gaseous nature of protein films at the air-water interface, often at areas as low as 1·5 sq.m./mg. Since the pressures exerted by corresponding films at the liquid-liquid interface at similar areas are considerably higher, it is difficult to conceive that these are not also in the gaseous state. The differences in the pressure-area isotherms for the two types of film at low pressures could be accounted for by different states of association of the molecules, different orientations of the molecules in the interface, or a combination of these and other factors. It was in an endeavour to elucidate this matter that the present investigation was made.

EXPERIMENTAL

Apparatus. In view of the fact that the author (Cheesman, 1946*a*) had already applied the Wilhelmy hanging-plate technique with some success to the study of protein films at the liquid-liquid interface, it was decided to attempt its adaptation to the present end. The apparatus is shown diagrammatically in Fig. 1. The trough was a cylindrical glass dish of about 12 cm. diam. and 3 cm. depth. It was accommodated in an adapted copper water oven which in turn was mounted in a stout wooden box. On the top of this rested a sensitive analytical balance. To the outer jacket of the oven were soldered inlet and outlet tubes to permit a constant flow of water pumped from a thermostat bath. Two tubes of 3 mm. diam. were soldered through the jacket. Through one of these passed wires from an external transformer to provide current for a flash-lamp bulb mounted on the roof of the oven. The other was arranged to correspond with a hole in the base of the balance case. Through this hole and tube passed a silk thread terminating at its upper end on a hook on the left-hand balance pan and at its lower end in a small wire hook on which the hanging plate could be suspended. A thick Perspex window was fitted in the door of the oven and in this was made a hole, oblique in the downward sense, to fit the barrel of an 'Agla' micrometer syringe. This hole was normally closed with a rubber bung. A thermometer, reading to 0·1°, stood inside the oven. A wad of

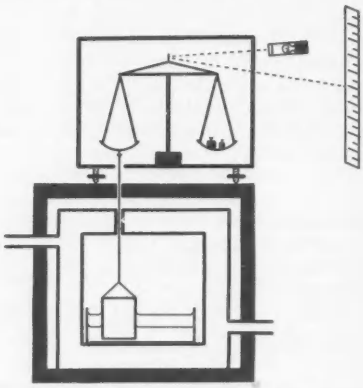

Fig. 1. Diagram of the interfacial film balance.

cotton wool, saturated with the non-aqueous phase of the two-phase system, was placed in the oven during experiments. This served to saturate the gaseous phase with the vapour and to minimize the error due to evaporation from the trough. The hanging plate was a microscope cover-slip ($4 \times 3 \times 0{\cdot}015$ cm.), to the upper corners of which a loop of silk thread was attached by sealing wax. The beam of the balance carried at its centre a concave mirror. An optical lever of 150 cm. length magnified the movements of the beam.

Procedure. The balance was calibrated by adding weights to the right-hand pan when the plate was hanging in a clean water surface. From the data thus obtained and the dimensions of the slide, the factor was calculated for converting the readings of the light spot into dynes/cm. The sensitivity of the balance was such that a change in surface tension of 1 dyne/cm. resulted in the deflexion of the light spot by about 3 cm. It was thus possible to measure surface pressures with an error of somewhat less than $\pm 0{\cdot}01$ dyne/cm.

An experiment was performed in the following manner. The glass dish was cleaned in hot chromic-sulphuric acid and washed with dilute phosphoric acid and distilled water. It was then transferred to the copper oven with crucible tongs. The aqueous phase was added to a depth of 1 cm. and the water surface was cleaned by suction. When benzene was used as oil phase, this cleaning process could be supplemented by using a drop of benzene as a piston to force contaminants to the side of the dish (Alexander & Teorell, 1939). A layer of oil phase of about 1 cm. thickness was then poured into the trough. Both liquids had been stored in the water thermostat so that undue time might not elapse in the attainment of temperature equilibrium. The glass plate was cleaned in its effective region with hot chromic-sulphuric

acid, washed with phosphoric acid and distilled water and dried in a blast of hot filtered air. It was then connected to its suspension and placed in position in the oil-water interface. The door of the oven was closed and the equilibration allowed to proceed until the position of the light spot was constant over 5 min. During the equilibration the sensitivity of the apparatus was controlled by the addition of weights to the right-hand balance pan. If the sensitivity proved to be identical with that when a clean water surface was employed, it could be assumed that the contact angle between the glass and the water at the oil-water interface was zero. If this did not prove to be the case, the plate was contaminated and it was necessary to recommence the experiment.

Protein was spread from 0·05% (w/v) solution in 60% (v/v) *iso*propanol, 0·5 M with respect to sodium acetate. The spreading of protein from such solvents has been shown to be rapid and complete, even against considerable surface pressures, at both the air-water and benzene-water interfaces (Ställberg & Teorell, 1939; Alexander & Teorell, 1939).

Additions of protein to the interface were made by passing the micrometer syringe through the hole in the Perspex window and, with the needle tip in the oil phase, applying drops of the solution to the water surface. After each such addition of about 2 μl. an interval of 2 min. was allowed to elapse before a surface pressure reading was taken. This allowed for the dispersion of the interfacial accumulation of propanol which was found in blank experiments to have no sensible effect upon the results when this precaution was observed. About twenty surface pressure readings were made over the range 0–1 dyne/cm. The corresponding areas were calculated from the amount of protein spread and the cross-sectional area of the trough.

Materials and methods. All inorganic reagents were of analytical quality and free from surface-active impurities. The water employed was distilled water from the laboratory supply redistilled from alkaline permanganate in a Pyrex apparatus. The benzene and *cyclo*hexane were of analytical grade and redistilled in a Pyrex apparatus.

The bovine serum albumin was a crystallized specimen supplied by Armour and Co. A specimen of pure human carboxyhaemoglobin was kindly presented to the author by Dr B. Cinader of the Lister Institute.

The albumin was weighed out after drying in a thin layer for 24 hr. *in vacuo* over H_2SO_4. The carboxyhaemoglobin was provided in 4·0% (w/v) solution in water. The concentration was confirmed by surface film measurements in the Langmuir trough (cf. Jonxis, 1939).

RESULTS

Human methaemoglobin at the benzene-water interface

Carboxyhaemoglobin dissolved in the propanol-sodium acetate mixture undergoes rapid conversion to methaemoglobin in the presence of air. That denaturation does not take place is evidenced by the fact that it is an easy matter to reconstitute the carboxyhaemoglobin by reduction with sodium hydrosulphite and subsequent passage of carbon monoxide. The changes in the prosthetic group are readily followed spectroscopically.

The points for six determinations on methaemoglobin at the interface between benzene and 0·001 M-sodium phosphate buffer (pH 6·8) at 25° are shown in Fig. 2. The full-drawn curve is drawn through the points representing the arithmetic means at ten arbitrary pressures. The *FA-F* curve appears to consist of two rectilinear sections, with a change of slope at about 0·4 dyne/cm. Extrapolation of the lower portion to $F = 0$ gives $\beta = 0{\cdot}24$ and the molecular weight $M = 10{,}200$. The disposition of the points suggests, however, that the linear relationship does not hold at pressures below

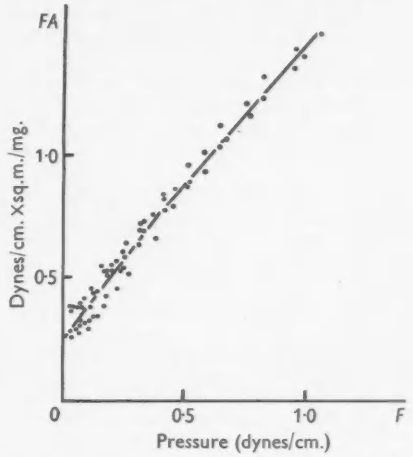

Fig. 2. *FA-F* curve for human methaemoglobin at the interface between benzene and 0·001 M-sodium phosphate buffer, pH 6·8, at 25°.

0·1 dyne/cm. A more probable extrapolation gives $\beta = 0{\cdot}28$–$0{\cdot}32$, and $M = 8200 \pm 400$. The slope of the lower portion of the curve, α in Eqn. 3, gives 1·33 sq.m./mg. for the area occupied by the protein in the uncompressed state. The upper portion of the curve gives $\alpha = 1{\cdot}16$ sq.m./mg. It seems probable that the change of slope at 0·4 dyne/cm. marks a transition between two types of orientation of the molecules in the surface.

Bovine serum albumin at the benzene-water interface

The results obtained with six films of bovine serum albumin at the interface between benzene and 0·001 M-sodium phosphate buffer (pH 6·8) at 25° are shown in the *FA-F* plot in Fig. 3. The full-drawn curve represents the arithmetic mean of the individual curves calculated for nine arbitrary pressures. The presence of a minimum appears to be well established at a pressure (0·2 dyne/cm.) corresponding to that observed by Bull (1947) with zein films at the air-water interface. Extrapolation

of the curve to zero pressure is rendered difficult by its form, but it seems probable that the value for β lies in the range 0·52–0·60, which gives an apparent molecular weight of 4400 ± 300. It is interesting to adduce Bull's comment to his zein curves that the minimum might be due not to attractive forces between the molecules in the film, but rather to the expulsion with increasing surface pressure into the aqueous phase of low-molecular material. There would seem at present to be no means of deciding between these alternatives. Analogous determinations at the air-water interface, described below, gave no evidence of a minimum and it seems hardly probable that impurities in the serum albumin can have been responsible for the effect.

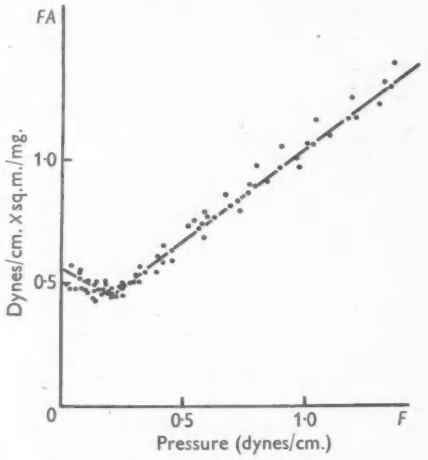

Fig. 3. *FA-F* curve for bovine serum albumin at the interface between benzene and 0·001 M-sodium phosphate buffer, pH 6·8, at 25°.

The slope of the rectilinear part of the curve gives a value of 0·75 sq.m./mg. for the area occupied by the protein in the uncompressed state.

Bovine serum albumin at the air-water interface

The apparent extensive dissociation of bovine serum albumin at the benzene-water interface made it desirable to investigate whether the protein spread from propanol solution would exhibit its native molecular size at the air-water interface. Appropriate experiments were therefore carried out with the same technique. Difficulty was found, however, in maintaining a zero contact angle between the surface and the hanging plate in the low-pressure region. Thus, although it was found that the apparent molecular weight was much higher under these conditions, it was thought unjustified to make an exact calculation. Bull (1950), however, encountered similar difficulties with egg albumin films and found that the contact angle righted itself at pressures above 0·5 dyne/cm., so that an extrapolation of the rectilinear part of the *FA-F* curve gave a reasonable value for the molecular weight. An examination of our results in the light of Bull's findings showed that the *FA-F* plot was, in fact, quite rectilinear at pressures above 0·15 dyne/cm. and gave a molecular weight value of $70{,}000 \pm 3000$. As this corresponds closely with the osmotic pressure value for the protein (Adair & Adair, 1930), the serum albumin evidently does not undergo irreversible dissociation in propanol solution. The slope of the curve gave a value for α of 0·81 sq.m./mg., a figure much lower than that recorded by Bull (1950) for the same protein spread from aqueous solution on 5 % (w/v) ammonium sulphate (1·2 sq.m./mg.).

Protein films at the cyclo*hexane-water interface*

The interfacial tension between *cyclo*hexane and water (50 dynes/cm.) is higher than that at the benzene-water interface (35 dynes/cm.). In an attempt to determine whether the interfacial energy would influence the behaviour of a gaseous protein film, a large number of experiments were made in which the proteins were spread at the interface between phosphate buffer and *cyclo*hexane. The *FA-F* curves for bovine serum albumin and human methaemoglobin often corresponded within the limits of experimental error with those obtained at the benzene-water interface. Reproducibility, however, was not good, and the behaviour of the methaemoglobin films was especially erratic, values as low as 3000 sometimes being obtained for the molecular weight. No adequate explanation could be found for this. Every effort was made to eliminate contamination. Lability of the contact angle may have been a contributing factor, although this was not visually evident. Whether methaemoglobin does, in fact, undergo extensive dissociation at the *cyclo*hexane-water interface, under the influence of an unknown and hitherto unreproducible factor, must remain for the present an open question.

Films of heat-denatured proteins at the benzene-water interface

It has been shown by Ställberg (1939) that serum albumin in propanol-sodium acetate solution will undergo true heat-denaturation without simultaneously being precipitated. This has been confirmed for a number of proteins by the present author (Cheesman, 1946*c*; unpublished observations). It was clearly of interest to study the effect of such denaturation on films spread under the conditions of the present work. Bovine serum albumin and human methaemoglobin were accordingly spread at the benzene-phosphate buffer interface from solutions

previously heated to 90° for 10 min. Within the limits of experimental error, no significant differences were found between these and films spread from unheated solution.

DISCUSSION

On the assumption that a protein film, at infinite expansion, may be treated as a perfect gas, the above results suggest that protein molecules undergo dissociation at the oil-water interface into fragments of a surface-active nature. If we assume values of 66,000 and 70,000 for the molecular weights of human methaemoglobin and bovine serum albumin respectively, these fragments would seem to have a mean weight in the former case of about one-eighth and in the latter about one-sixteenth of that of the undissociated molecule at pH 6·8.

The author (Cheesman, 1946*b*) has previously found indications that unimolecular films of protein are oriented in different manners at the air-water and the oil-water interfaces, and this has recently been established by Davies (1951) in his ingenious measurements of the electrical phase boundary potentials of protein films at the petroleum ether-water interface. It may be that a more rigid orientation of non-polar side chains towards the oil brings about a disruption of secondary intramolecular linkages which remain unaffected at the air-water interface.

It is tempting to suggest a relation of the present finding with the now discounted hypothesis of Sørensen (1930) according to which the serum proteins, and many others, are systems of sub-molecular components linked by residual valencies and capable of reversible dissociation.

An interfacial dissociation of native proteins could clearly be of interest in relation to proteogenesis. There is little doubt that the biological synthesis of proteins takes place at phase boundaries within the cell. If protein molecules have a structure of sub-units in physical association, the question presents itself whether the final stage of the synthesis may be regarded as a specific adsorptive organization into a single entity of a number of enzymically synthesized precursors.

SUMMARY

1. A method has been devised for the study in the low-pressure region of unimolecular films of proteins at the interface between two liquids.

2. Films of human methaemoglobin and bovine serum albumin have been studied at the benzene-water interface.

3. The laws for a two-dimensional gas have been applied to the experimental data. It is concluded that the molecule of human methaemoglobin dissociates into about eight, and that of bovine serum albumin into about sixteen kinetic units when adsorbed at the benzene-water interface at pH 6·8.

This work was carried out during the tenure of a Senior Research Award of the Department of Scientific and Industrial Research. The author wishes to thank Prof. E. K. Rideal for many helpful discussions. Dr B. Cinader kindly provided the specimen of human carboxyhaemoglobin used in the experiments.

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The Inhibition of Chymotrypsin by Diethyl *p*-Nitrophenyl Phosphate

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The alkyl fluorophosphonates were first shown in 1940 (Adrian, Feldberg & Kilby, 1947) to be very powerful inhibitors of cholinesterase, and for a time they were thought to be quite specific for this enzyme. Later, other esterases, such as human milk lipase and liver esterase (Webb, 1948) and citrus acetylesterase (Jansen, Nutting & Balls, 1947) were also shown to be inhibited, although not to the same extent. The discovery that trypsin and chymotrypsin display esterase as well as proteolytic activity (Schwert, Neurath, Kaufman & Snoke, 1948; Kaufman, Schwert & Neurath, 1948) prompted the investigation of the action of fluorophosphonates on these enzymes (Jansen, Nutting, Jang & Balls, 1949), and it was found that both were sensitive to di*iso*propyl fluorophosphonate (DFP) and that the esterase and proteolytic activities were inhibited in parallel. A considerable number of other organic phosphorus compounds have been discovered which resemble the fluorophosphonates in being highly toxic and powerful anti-cholinesterases. Some of these are now being produced commercially as insecticides, *OO*-diethyl *O*-*p*-nitrophenyl thiophosphate (Parathion) being one of the best known. The increasing use of such toxic compounds makes a fuller knowledge of their mode of action desirable. It would also be valuable if one could eventually suggest, on a rational basis, the direction in which a search could most profitably be made for compounds of high insecticidal activity combined with low mammalian toxicity. It appeared to us that a suitable starting point for the investigation of the mode of action of organic phosphorus compounds would be a detailed study of a model system using an inhibitor whose chemistry is straightforward and an enzyme which could be obtained pure and crystalline, preferably of small and known molecular weight. As an inhibitor, we have selected diethyl *p*-nitrophenyl phosphate (E 600, Paraoxon), the oxygen analogue of Parathion, as the chemistry of the latter is complicated by isomerization and really pure material is very difficult to obtain. Chymotrypsin has been found to be a very suitable enzyme, and is interesting in possessing several types of enzymic activity. The enzymic hydrolysis of certain *p*-nitrophenyl esters has been investigated, and the inhibition of the proteolytic, esterase and amidase activities of chymotrypsin by E 600 has been studied.

EXPERIMENTAL

Materials

Chymotrypsin. Two samples of enzyme were used: crystalline bovine α-chymotrypsin supplied by Armour and Company of Chicago (sample *A*) and a similar specimen from Canada of unknown origin (sample *B*). Both had been obtained by recrystallization from magnesium sulphate solution. The proteolytic activities were determined by Anson's haemoglobin method (Anson, 1938), as used by Kunitz & Northrop (1935) and expressed as tyrosine units T.U.^{Hb}/mg. protein nitrogen (P.N.), where one T.U. is the amount of enzyme which will liberate the equivalent of 1 mmol. tyrosine/ml./min. at 35·5°. Kunitz & Northrop (1935) report a mean value of 0·039 T.U.^{Hb}/mg. P.N. for pure chymotrypsin recrystallized 8–10 times and containing 15·5% N. Owing to the small amount of material available, recrystallization of our samples to constant activity was not attempted, and the amounts of active chymotrypsin in them were calculated using the value given by Kunitz & Northrop for pure enzyme (Table 1).

Diethyl p-*nitrophenyl phosphate* (E 600). Diethylchlorophosphonate was added to a suspension of an equimolecular amount of anhydrous sodium *p*-nitrophenate in dry xylene and heated at 100–110° for 1 hr. After removal of salt and solvent, the oil was washed with Na_2CO_3, dried and distilled, b.p. 142°/6 × 10^{-3} mm. (Schrader, 1946).

Ethyl p-*nitrophenyl carbonate.* M.p. 66·5–67° (Ransom, 1898).

p-*Nitrophenyl acetate.* M.p. 79·5–80° (Kaufmann, 1909).

Table 1. *Chymotrypsin content of enzyme samples*

(T.U.^{Hb}/mg. P.N. defined above).

Sample	N analysis* (% in sample)	Protein (% in sample)	Proteolytic activity (T.U.^{Hb}/mg. P.N.)	Chymotrypsin in protein (%)	Chymotrypsin in sample (%)
A	11·5	74	0·026	67	49
B	14·1	91	0·028	71	65

* The non-protein nitrogen was less than 0·1%.

L-Tyrosine ethyl ester. M.p. 107° (Fischer, 1901).

N-acetyl L-tyrosine amide. M.p. 223° (Kaufman & Neurath, 1949).

Methods

Measurement of proteolytic activity. This was measured by a haemoglobin digestion method similar to that described by Anson (1938) for the assay of the proteolytic activity of trypsin. A standard solution of denatured haemoglobin was prepared from whipped ox blood. This substrate (5 ml.) was incubated at pH 7·5 and 35·5° with 1 ml. of enzyme solution for exactly 10 min. The reaction was then stopped, and unchanged protein precipitated, by shaking with 10 ml. of 0·3 N-trichloroacetic acid. After standing for 30 min. the reaction products were filtered through Whatman no. 1 paper. 0·5 N-NaOH (10 ml.) and 3 ml. Folin & Ciocalteu's (1927) phenol reagent were added to 5 ml. of filtrate, and the colour which developed was compared within 5–10 min. with that from a standard solution of tyrosine by means of the 'Spekker' photoelectric absorptiometer using orange filters (O.Y. 2). Blanks were prepared for each determination by mixing 1 ml. enzyme solution with 10 ml. 0·3 N-trichloroacetic acid before adding 5 ml. of substrate, and the colour developed and measured as before. A calibration curve for the instrument was plotted, and a linear relationship found to hold between the drum reading and tyrosine concentration over the range 1–8 mmol.-tyrosine/5 ml. solution. There was also a linear relationship between the concentration of enzyme incubated with substrate and the colour subsequently developed in the filtrate over the range 0·01–0·10 mg. enzyme sample/ml. enzyme solution.

During inhibition by diethyl *p*-nitrophenyl phosphate (E 600), nitrophenol is liberated, but experiments showed that at the concentrations observed it has no effect on either the digestion or colour development stage of this determination.

Assay of p-*nitrophenol.* The absorption spectrum of nitrophenol in alkaline solution was found to show a peak at 4000 A. due to the nitrophenate ion. The optical density at this wavelength, measured on the Unicam Quartz Spectrophotometer, was used to estimate nitrophenol concentrations. Glass cells $10 \times 10 \times 40$ mm. were used. A calibration curve of optical density against nitrophenol concentration over a range 2×10^{-6} to 6×10^{-5} M in 0·1 M-phosphate buffer, pH 7·60, and containing 5% *iso*propanol, was plotted and used in subsequent estimations (5% *iso*propanol had no effect on chymotrypsin activity during incubation at 25° for 18 hr.).

When the nitrophenol liberated in solutions containing chymotrypsin and a *p*-nitrophenyl ester was being measured, blank solutions identical except that no enzyme was present were prepared and incubated in parallel.

During runs in which the liberation of nitrophenol was being followed kinetically, the glass cells with lids were kept at the bottom of boiling tubes deeply immersed in a thermostatically controlled bath. The tubes contained a little cotton wool and water such that the level of liquid inside and outside the glass cell was the same. At intervals, the cells were removed from the tubes, dried and the optical densities measured as rapidly as possible.

Hydrolysis of L-tyrosine ethyl ester (TEE). The rate of hydrolysis of TEE was measured by a continuous potentiometric titration method similar to that of Schwert *et al.* (1948), whereby the acid liberated during hydrolysis was continuously titrated with dilute alkali to constant pH as measured on a Marconi pH meter.

The electrodes dipped into 25 ml. of a 0·025 M solution of TEE, pH 6·20, contained in a beaker in a bath at 25° and stirred by a stream of N_2. Enzyme solution (1 ml.) was added, and 0·02 N-NaOH was run in in portions of 0·50 ml., the time when the pH meter registered pH 6·20 being noted. The pH varied between 6·15 and 6·30 during the hydrolysis.

Unbuffered solutions were found preferable with this dilute alkali, and the reference pH 6·20 was chosen to give the optimum pH of 6·25 during the hydrolysis as quoted by Jansen *et al.* (1950).

Hydrolysis of N-acetyl L-tyrosine amide (ATA). This was followed by a method similar to that of Schwert *et al.* (1948), whereby the NH_3 liberated at intervals during the hydrolysis is measured by a modification of the Conway microdiffusion method.

A solution of chymotrypsin in 0·1 M-phosphate buffer, pH 7·60, was mixed with a solution of 0·04 M-ATA in buffer containing 10% *iso*propanol and incubated at 25°. Samples (0·2 ml.) were withdrawn at intervals and introduced into the outer rings of Conway plates. Saturated K_2CO_3 (1 ml.) was added to the outer rings to volatilize the NH_3, and the plates were tipped and mixed, the NH_3 being absorbed in the inner chambers in 1% boric acid plus indicator. The plates were left at room temperature overnight before titrating with 0·01 N-HCl from a Conway microburette. This acid had previously been calibrated against standard $(NH_4)_2SO_4$ solutions under the same conditions. The period of digestion was taken as the time from addition of substrate until the mixing of the sample with alkali. Blanks were carried out by placing 0·1 ml. of enzyme solution and 0·1 ml. of ATA solution apart in the outer rings of the plates and mixing only after addition of the saturated K_2CO_3. Constant low blanks were obtained. The spontaneous hydrolysis of ATA under these conditions was negligible.

RESULTS

Inhibition of chymotrypsin by E 600

E 600 inhibits the proteolytic activity of crystalline chymotrypsin. Various concentrations of E 600 in *iso*propanol were added to a solution of chymotrypsin in 0·01 M-phosphate buffer, pH 7·60. The final concentrations were: chymotrypsin (sample *B*), 1 mg./ml.; E 600, 10^{-4} to $1{\cdot}5 \times 10^{-3}$ M; *iso*propanol, 5%.

The solutions were incubated for either 254 or 423 min. at 25° and the proteolytic activity then determined by the method given in the experimental section. A number of experiments showed that there was negligible change in activity of control solutions (without inhibitor) incubated under the same conditions for 24 hr. The logarithm of the percentage residual activity was then plotted against the inhibitor concentration (Fig. 1). After 423 min. incubation under the conditions used, a 50% inhibition of enzyme was given by 3×10^{-4} M-E 600. Tests were carried out on the reversibility of this inhibition.

The rate of inhibition by 10^{-3} M-E 600 of a solution of chymotrypsin at pH 7·60 at 25° was measured in

the presence of a concentration of nitrophenol one hundred times that liberated during the inhibition. Control experiments showed that such concentrations had negligible effect on the estimation of proteolytic activity by Anson's method. No differences in activity were found between samples withdrawn at various times from a solution containing enzyme, inhibitor and nitrophenol, and a control containing only enzyme and inhibitor.

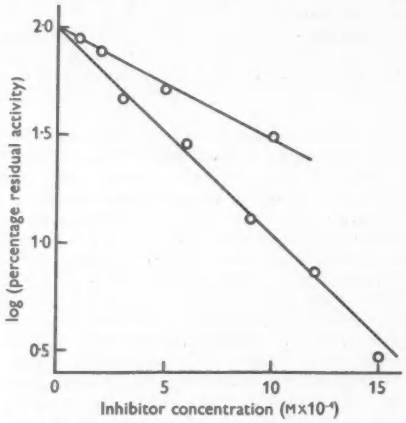

Fig. 1. Inhibition of proteolytic activity of chymotrypsin by E 600 at 25° after incubation for 254 min. (upper line) and 423 min. (lower line). Chymotrypsin concentration, 1 mg./ml. (sample *B*).

A further check on the irreversibility of the inhibition was obtained by testing the activity of a solution of chymotrypsin which had been completely inhibited by reaction with 10^{-3} M-E 600 for 24 hr. at 25°, and then dialysed against 0·005 N-sulphuric acid at 5° for 26 hr. The proteolytic activity of this sample, estimated by Anson's method, was compared with that of the original solution of chymotrypsin which had been incubated and dialysed in parallel. The activity of the inhibited sample was 3 % of the control, which is less than the experimental error of the determination.

Hydrolysis of p-*nitrophenyl carbonate (NPC) and acetate (NPA)*

These substances were investigated as possible substrates for chymotrypsin. Solutions of the substrate were made up in *iso*propanol to give the required final substrate concentrations in 5 % *iso*propanol when added to a solution of the enzyme in 0·1 M-phosphate buffer, pH 7·60. The rate of liberation of nitrophenol was measured by the rate of increase of optical density at 4000 A. Preliminary experiments indicated that the substrates were hydrolysed 200 times more quickly at 25° at pH 7·60 in the presence of 1 mg./ml. chymotrypsin than in its absence. The rate of spontaneous hydrolysis was constant over the observed period and proportional to the substrate concentration. The first-order velocity constants (k) were $9{\cdot}2 \times 10^{-4}$ min.$^{-1}$ for the acetate and $3{\cdot}5 \times 10^{-4}$ min.$^{-1}$ for the carbonate. The latter was used in most experiments to reduce corrections for spontaneous hydrolysis.

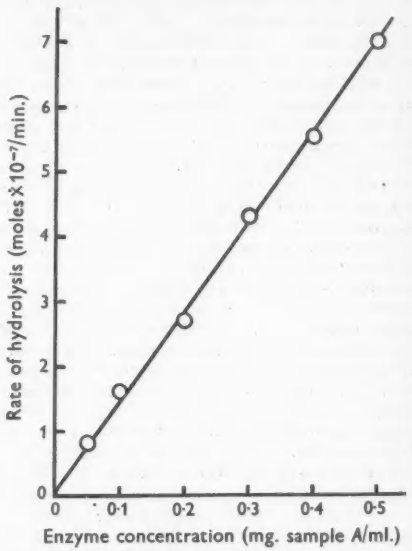

Fig. 2. Relationship between the rate of enzymic hydrolysis of *p*-nitrophenyl ethyl carbonate (at 25° and pH 7·6) and the concentration of chymotrypsin present. (Substrate concentration, 10^{-3} M.)

The rate of hydrolysis of NPC was found to be proportional to chymotrypsin concentration (Fig. 2). The concentration of NPC was 10^{-3} M. The average activity found was $2{\cdot}86 \times 10^{-6}$ moles/min./mg. pure chymotrypsin giving a turnover number of 78·6 molecules substrate/min./molecule chymotrypsin. The initial rates of enzymic hydrolysis of NPC were independent of substrate concentration over the range 5×10^{-4} to $2{\cdot}5 \times 10^{-5}$ M, but at the latter concentration the rate of hydrolysis ceased to be linear after 8 min. and hydrolysis was complete after 15 min. Since the initial velocity of hydrolysis was constant, except at very low substrate concentrations, it was found desirable to use a 'progress curve' method to determine the Michaelis constant (K_m). A solution of chymotrypsin in M/15 phosphate buffer, pH 7·60, was diluted with NPC in buffer containing *iso*propanol to final concentrations of chymotrypsin (65% pure), 1 mg./ml.; NPC,

3×10^{-5} M; *iso*propanol, 5 %. The rate of hydrolysis was determined by the optical density method.

Tangents to the hydrolysis curve were constructed where it began to depart from a straight line (Fig. 3), giving the rate of hydrolysis (V). The substrate remaining (S) was calculated by subtracting the nitrophenol concentration at that time from the infinity value. A plot of $1/V$ against $1/S$ (Fig. 4) gave a straight line from which K_m was found to be $1{\cdot}3 \times 10^{-6}$ M from the equation

$$\frac{1}{V} = \frac{K_m}{V_0}\frac{1}{S} + \frac{1}{V_0}$$ (Lineweaver & Burk, 1934).

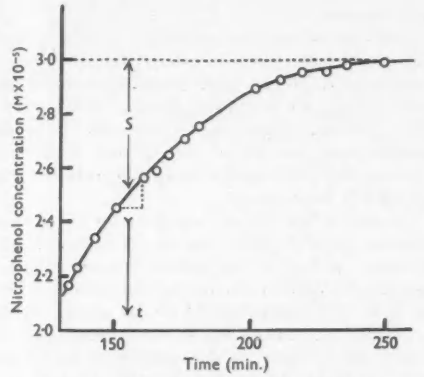

Fig. 3. Later (non-linear) portion of hydrolysis curve of *p*-nitrophenyl ethyl carbonate by chymotrypsin. The tangent to the curve at time *t* gives the rate of hydrolysis V at substrate concentration S. Chymotrypsin concentration, 1 mg./ml. (sample *B*).

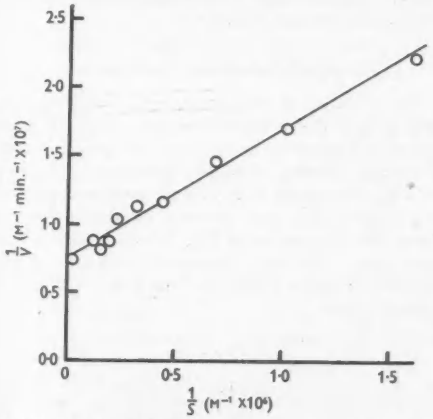

Fig. 4. Velocities of hydrolysis by chymotrypsin (V) of *p*-nitrophenyl ethyl carbonate at 25°, pH 7·6, at various substrate concentrations (S) plotted as reciprocals to obtain K_m.

Similar measurements were made with NPA and the approximate values of K_m and turnover number were 3×10^{-3} M and 920 molecules/min./molecule chymotrypsin respectively.

Amount of nitrophenol liberated after complete inhibition of chymotrypsin (ChTr) *by E* 600

Table 2 gives the results of a series of experiments in which the concentration of nitrophenol was measured by the optical density method after the proteolytic activity of the enzyme had been completely inhibited. The 'infinity' value was measured after 18–27 hr. incubation. It remained constant for several hours.

Table 2. *Observed and calculated concentrations of nitrophenol after complete inhibition of chymotrypsin by E* 600

(Nitrophenol observed = 96·7 % of theoretical (mean of 13). The theoretical nitrophenol concentrations were calculated assuming Eqn. 1, enzyme purities of 49 and 65 % for samples *A* and *B* respectively, and a mol.wt. for chymotrypsin of 27,000.)

Concn. of ChTr (mg./ml.)	Concn. of E 600 (M x 10^{-4})	Observed nitrophenol (M x 10^{-5})	Theoretical nitrophenol (M x 10^{-5})
1·0	10	1·64 (mean of 5)	1·82
(sample *A*)	5	1·75	1·82
1·5	15	3·62 (mean of 2)	3·61
(sample *B*)	10	3·54 (mean of 3)	3·61
	7·5	3·70 (mean of 2)	3·61

The purity of the enzyme was calculated by comparing the proteolytic activity at 35·5° with that given by Northrop for the pure enzyme. The last column gives the theoretical concentration of nitrophenol calculated from the equation

$$\underset{(a-x)}{\text{ChTr—H}} + \underset{\text{(I)}}{\text{NO}_2.\text{C}_6\text{H}_4.\text{O.PO.(OEt)}_2} \xrightarrow[k_i]{} \underset{(x)}{\text{ChTr—PO.(OEt)}_2} + \underset{(x)}{\text{NO}_2.\text{C}_6\text{H}_4.\text{OH}}, \quad (1)$$

assuming a molecular weight of 27,000 for the enzyme (Jansen, Nutting & Balls, 1949). Denatured chymotrypsin does not react with E 600 according to Eqn. 1. A solution of chymotrypsin in 0·0025 N-hydrochloric acid was divided into two; one portion was partly denatured by heating at 100° for 15 min. Samples of both solutions were incubated at 25° and pH 7·60 with 10^{-3} M-E 600, and the nitrophenol liberated after 22 hr. was measured. The proteolytic activities of the denatured and control enzyme solutions were measured after incubating overnight at 25°. The denatured enzyme showed a fall in proteolytic activity to 72 % of that of the control, while the nitrophenol which it liberated from E 600 was 68 % of that liberated by the control. Thus the 28 % of denatured protein formed does not react with E 600 to liberate nitrophenol.

Rate of inhibition of chymotrypsin with various substrates

Simultaneous estimations of the rate of inhibition of proteolytic activity and the rate of liberation of nitrophenol were made as in a previous paper (Hartley & Kilby, 1950). Chymotrypsin solutions in 0·1 M-phosphate buffer, pH 7·60, were incubated at 25° with E 600, the final concentrations being: ChTr, 1·0 mg./ml.; E 600, 10^{-3} M; *iso*propanol, 5 %. The proteolytic activity was measured at various times by the haemoglobin method and the rate of liberation of nitrophenol by the optical density method. The results of a number of such experiments are indicated in Fig. 5. It can be seen that the proteolytic activity is inhibited at the same rate as nitrophenol is liberated.

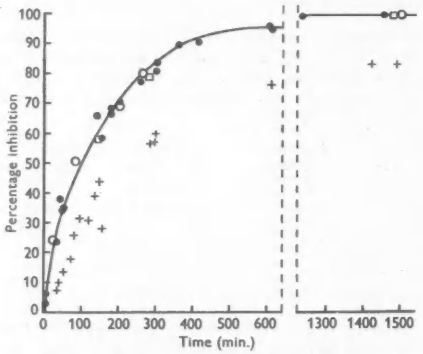

Fig. 5. Rate of inhibition of various enzymic activities of chymotrypsin by 10^{-3} M-E 600 at 25°, pH 7·6. Percentage inhibition of proteolytic activity, ● (Hb); of amidase activity, ○ (ATA); of amino-acid esterase activity, □ (TEE); and of nitrophenyl esterase activity, + (NPC). The curve is the rate of liberation of nitrophenol (as percentage of 'theoretical'), calculated from Eqn. 1, using the observed velocity constant, $k_i = 5{\cdot}3$.

The rate of inhibition of the enzymic hydrolysis of TEE was compared with the rate of liberation of nitrophenol. A solution of chymotrypsin at pH 7·60 was incubated at 25° with 10^{-3} M-E 600 and the rate of inhibition estimated by withdrawing samples at various intervals and measuring the rate of hydrolysis of 0·025 M-TEE by the potentiometric titration method after suitable dilution of the inhibited enzyme. The results obtained, plotted in Fig. 5, indicate that the hydrolysis of TEE by ChTr is inhibited at the same rate as the proteolytic activity and bears the same relation to the rate of liberation of nitrophenol. From the initial rate of hydrolysis, the turnover number was found to be 1500 molecules substrate/min./molecule enzyme.

Chymotrypsin was inhibited by 10^{-3} M-E 600 under the same conditions as before. Samples were withdrawn at intervals and after suitable dilution were incubated at 25° with 10^{-3} M-NPC in 0·1 M-phosphate buffer, pH 7·60, containing 5 % *iso*propanol. The rate of hydrolysis was measured by the change in optical density at 4000 A. The rate of inhibition of this esterase activity is shown in Fig. 5. The rate of inhibition of hydrolysis of NPC does not coincide with the inhibition of other activities of chymotrypsin.

Solutions of chymotrypsin in 0·1 M-phosphate buffer, pH 7·60, were incubated at 25° in the presence of E 600 and *iso*propanol, final concentrations being: ChTr (43 % pure), 3·00 mg./ml.; E 600, 10^{-3} M; *iso*propanol, 5 %. At intervals, samples were withdrawn and diluted with equal volumes of a solution of 0·04 M-ATA in buffer containing 5 % *iso*propanol.

The rate of liberation of ammonia at 25° in these mixtures was followed by the Conway micro-diffusion technique described above. Control experiments carried out in parallel indicated that the rate of hydrolysis of ATA by chymotrypsin under these conditions was linear, corresponding to a turnover number of 4·8 molecules ATA/min./molecule pure ChTr. The rate of liberation of ammonia in the various inhibited samples compared to that of the control gave the percentage inhibition. The rate of inhibition of amidase activity is shown in Fig. 5. The amidase activity of chymotrypsin is inhibited at the same rate as the proteolytic and amino-acid esterase activity.

Kinetics of liberation of nitrophenol

The kinetics of the reaction between chymotrypsin and E 600 were studied by following the rate of liberation of nitrophenol by measurements of optical density changes. Solutions in buffer, pH 7·6, containing 0·5–0·7 mg. pure enzyme/ml., 5 % *iso*propanol and various concentrations of E 600 were incubated at 25°. If the inhibitor concentration (I) is large compared with that of the enzyme (a) and a reaction of the form of Eqn. 1 is assumed, then

$$\frac{dx}{dt} = k_i I\,(a-x),$$

i.e.
$$t = \frac{2{\cdot}303}{Ik_i} \log \frac{a}{(a-x)}. \qquad (2)$$

The concentration of nitrophenol observed at time t is x, while that after complete inhibition (about 18 hr.) is a. In Fig. 6, $\log 100\,(a-x)/a$ is plotted against t, for various inhibitor concentrations. The straight line plots support a reaction of the form postulated above, and the velocity constant of inhibition (k_i) can be calculated from their slopes. Values of k_i obtained in this way from the data of Fig. 6 are given in Table 3.

Kinetics of inhibition of proteolytic activity

A check on the identity of the rate of inhibition of proteolytic activity and the rate of liberation of nitrophenol is given by the results plotted in Fig. 1.

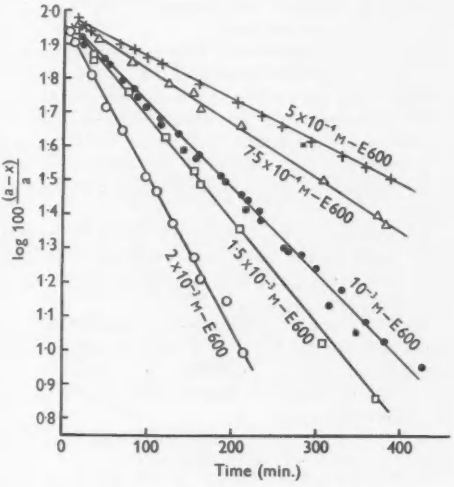

Fig. 6. Rate of liberation of nitrophenol from chymotrypsin and various concentrations of E 600 at 25°, pH 7·6.

Table 3. *Velocity constant for reaction of chymotrypsin and E 600*

Concn. of E 600 ($M \times 10^{-4}$)	$\frac{d}{dt} \log \frac{100\,(a-x)}{a}$	k_i (litres moles^{-1} min.$^{-1}$)
5	0·124	5·7
7·5	0·158	4·9
10	0·246	5·7
15	0·299	4·6
20	0·457	5·3

Since $100\,\frac{(a-x)}{a}$ is the percentage residual proteolytic activity, the velocity constant of inhibition (k_i) can be calculated from the slopes of the two lines using Eqn. 2. Values of 5·0 and 5·3 for k_i are obtained from the 245 and 423 min. incubation curves respectively, in satisfactory agreement with the values of k_i in Table 3, which are derived from nitrophenol liberation measurements.

DISCUSSION

There is an increasing amount of evidence to show that chymotrypsin contains only a single active centre, and that this is responsible for the various different types of enzymic activity shown by the enzyme.

Di*iso*propyl fluorophosphonate (DFP) inhibits chymotrypsin in such low concentration that the enzyme and inhibitor must be present in about equimolecular amounts. It has been shown (Jansen, Nutting & Balls, 1949; Jansen *et al.* 1950) that when chymotrypsin is completely inhibited by DFP containing ^{32}P, the inactivated enzyme can be obtained crystalline and contains about one atom of phosphorus and two *iso*propoxy groups per molecule; there is some evidence that the fluorine atom of the inhibitor is eliminated as hydrogen fluoride. No combination between inhibitor and protein could be demonstrated when DFP was incubated with chymotrypsinogen, the precursor of the enzyme, in which the 'active centre' is presumably 'masked'.

The results obtained from our studies on the inhibition of chymotrypsin by E 600 support the hypothesis that this enzyme contains one grouping only which can combine with the phosphorus group of inhibitors. The amount of nitrophenol liberated when chymotrypsin has reacted completely with E 600 is shown in Table 2 and the close correspondence between the observed concentrations and those calculated for a reaction such as

$$\mathrm{ChTr{-}H + (EtO)_2PO.C_6H_5.NO_2 = ChTr{-}PO{-}(OEt)_2 + NO_2.C_6H_4.OH}$$

supports this type of reaction.

The rate of appearance of nitrophenol in a solution containing an excess of inhibitor satisfies first-order kinetics as would be expected from the above reaction; the first-order velocity constant k_i which was obtained from nitrophenol measurements also satisfied the kinetics of proteolytic inhibition (Fig. 5).

The inhibition cannot be reversed by the addition of excess nitrophenol during the reaction. Dialysis of inhibited enzyme failed to regenerate significant activity. It may be concluded that the inhibition is irreversible, in the sense that the reaction with inhibitor proceeds only to the right, although a slow reactivation by hydrolysis of the $-O.PO(OEt)_2$ group attached to the enzyme (such as that reported by Wilson (1951) in the inhibition of cholinesterase by tetraethyl pyrophosphate) cannot be totally excluded.

Many workers believe that the inhibitory power of the organic phosphorus group of compounds depends on the presence of an easily hydrolysed P—X bond in molecules such as $(RO)_2POX$ or $(NMe_2)_2POX$, combined with an electrophilic phosphorus atom. A type of phosphorylation mechanism, as in Eqn. 1, is in keeping with the nature of the inhibitors. The single centre appears to be responsible for the various enzymic activities of chymotrypsin. The proteolytic activity (measured by the haemoglobin method), the amino-acid

esterase and amidase activities, as exemplified by the hydrolysis of L-tyrosine ethyl ester and acetyl L-tyrosinamide respectively, were all found to be inhibited at exactly the same rate as nitrophenol was liberated. A very close correspondence between the centre involved and the site of attack of the inhibitor is indicated. Very stringent structural requirements for the specific substrates for chymotrypsin have been postulated by Schwert & Neurath (1950). The requirements for ester, amide and peptide substrates are very similar, again suggesting reaction at a common active centre. The organic phosphorus inhibitors do not conform to these structural requirements but do show a great affinity for the enzyme, and if, as we believe, they react at the active centre, it appears possible that non-phosphorus analogues might behave as substrates rather than as inhibitors. A carbon analogue of E 600, *p*-nitrophenyl ethyl carbonate, was tried and found to be non-inhibitory, and to be enzymically hydrolysed, although the turnover number was rather lower than that for L-tyrosine ethyl ester. *p*-Nitrophenyl acetate was also a substrate, and was hydrolysed rather more readily.

The inhibition of the *p*-nitrophenyl esterase activity was anomalous, in that it did not follow the same curve as those for the other enzymic activities (Fig. 5), and when the latter had been completely inhibited, about 20 % of the nitrophenyl esterase activity remained. The reason for this difference is not clear at present. This anomalous behaviour is being investigated further.

SUMMARY

1. The proteolytic activity of chymotrypsin is inhibited by diethyl *p*-nitrophenyl phosphate (E 600). 50 % inhibition is produced by 3×10^{-4}M-E 600 after 423 min. incubation at pH 7·6 at 25°. The inhibition is irreversible.

2. *p*-Nitrophenyl ethyl carbonate and *p*-nitrophenyl acetate are substrates for chymotrypsin. Values of K_m and turnover number are obtained.

3. *p*-Nitrophenol is liberated during inhibition of chymotrypsin by E 600. The molar concentration of nitrophenol liberated after complete inhibition is equivalent to the molar concentration of the enzyme.

4. The rates of inhibition by E 600 of the proteolytic, amidase and amino-acid esterase are equal and exactly correspond to the rate of liberation of nitrophenol during the inhibition.

5. The kinetics of liberation of nitrophenol are explained on the basis of a bimolecular reaction between enzyme and inhibitor. The velocity constant obtained satisfies the kinetics of inhibition of proteolytic activity.

6. The results support the hypothesis of a single active centre in chymotrypsin.

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Gas-liquid Partition Chromatography: the Separation and Micro-estimation of Volatile Fatty Acids from Formic Acid to Dodecanoic Acid

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Industry has long used charcoal or other solid absorbents in columns for cleaning gas streams or for solvent recovery, and more recently Turner (1943), Claesson (1946), Glueckauf, Barker & Kitt (1949), Phillips (1949) and Turkel'taub (1950) have used charcoal in gas chromatograms for the analysis of hydrocarbons and esters. Gas-liquid scrubbing columns have been used in industry for many years but, though Martin & Synge (1941) suggested the use of gas-liquid partition chromatograms for analytical purposes, no work has been reported along these lines.

This paper presents modifications necessary to the theory of Martin & Synge (1941) to allow for the compressibility of the mobile phase and describes the application of the gas-liquid partition chromatogram to the separation of volatile fatty acids. The separations obtainable by this method are essentially parallel to those obtainable by distillation, but good separations can be achieved much more easily and it is possible to work with very much smaller quantities. In fact, the lower limit of quantity of material used is determined only by the efficiency of detection. In general, far less trouble from azeotrope formation is to be expected, since the concentrations of the substances to be separated in the liquid phase are always low and it may be possible to choose a liquid phase which associates only with one component of the azeotrope. Work on a preparative scale, though theoretically possible, is likely to be inconvenient because of the bulk of the apparatus. The method of detection described here is acid-base titration, but many methods of detecting changes in the composition of a gas stream could be used and it is intended in future publications to explore some of these, which should extend the range of application to all substances capable of being distilled at the pressure of a few mm. of mercury.

In suitable cases the gas-liquid partition column has two principal advantages over the ordinary liquid-liquid partition column: (*a*) the low viscosity of the mobile phase allows relatively longer columns to be used with a corresponding gain in efficiency and (*b*) in general it is easier to detect changes in composition of a gas than of a liquid stream.

A THEORY OF GAS-LIQUID PARTITION CHROMATOGRAPHY

The theory of gas-liquid chromatography differs from that of liquid-liquid chromatography, where a constant partition coefficient is assumed (Martin & Synge, 1941), only by virtue of the fact that the mobile phase is compressible and thus produces a gradient of gas velocity down the column. It is assumed that the partial pressures of the substances to be separated are negligible in relation to that of the carrying stream of gas. Let

p_1 = pressure of gas applied to chromatogram.
p_0 = pressure of gas at outlet.
p = pressure of gas at point distant x from the outlet.
v = linear velocity of gas at point x.
l = length of column.
t = time which elapses before the centre of the zone emerges from the column.
F = volume of gas emerging from outlet in unit time.
V_R = tF = the retention volume of the centre of the zone.
V_R^0 = limiting value of V_R as p_1/p_0 tends to unity.
a = the area occupied by the gas phase in any cross-section of the column.
K = column constant, a function of the viscosity of the gas phase and the tightness of the column packing.

Then
$$K\frac{dp}{dx}=\frac{Fp_0}{p}=av.$$

Integrating to give the pressure along the column:

$$K\frac{p^2}{p_0}=2Fx+Kp_0,$$

or
$$K=\frac{2Fxp_0}{p^2-p_0^2},$$

or
$$F=\frac{p_0K}{2x}\left[\left(\frac{p}{p_0}\right)^2-1\right]. \qquad (1)$$

Now
$$t=\int_0^l\frac{dx}{vR_F}=\int_0^l\frac{ap\,dx}{R_F\,p_0F}. \qquad (2)$$

Here R_F has the usual chromatographic meaning (Consden, Gordon & Martin, 1944), that is, it represents the rate of movement of the zone of the substance under consideration relative to the rate of flow of the moving phase. In the present case the rate of flow of the moving phase increases continuously as it advances down the column, but the R_F remains constant and the rate of movement of the zone increases correspondingly. The R_F is a constant depending only on the substance to which it refers, the temperature, the nature and amount of the stationary phase and, in so far as the support is not inert, on its amount.

Rewriting Eqn. 2 in terms of pressures,

$$t=\int_{p_0}^{p_1}\frac{Kap^2\,dp}{R_F F^2 p_0^2}=\frac{Ka\,(p_1^3-p_0^3)}{3R_F p_0^2 F^2},$$

$$V_R=Kap_0\,\frac{[(p_1/p_0)^3-1]}{3R_F F}.$$

Hence, by (1),

$$V_R=\frac{2}{3}\frac{al}{R_F}\frac{(p_1/p_0)^3-1}{(p_1/p_0)^2-1}. \qquad (3)$$

As p_1/p_0 tends to unity then V_R tends to $V_R^0=al/R_F$. In fact, the flow rate V is measured at room temperature and should be corrected to the column temperature.

Since the value of R_F is not dependent on p_0 it is not possible by reducing p_0 to reduce the temperature of operation of the column. Further, since the viscosity of the gas is independent of the absolute pressure, when p_0 is low, the ratio of the pressures at the two ends of the column becomes excessive if a reasonable rate of flow is to be maintained. Under normal conditions it is convenient for p_0 to be atmospheric pressure. It might, however, be preferable to increase p_0 by a factor of approximately 10 if a very long column is to be used in order to effect a difficult separation. This is due to the fact that the absolute pressure drop from one end of the column to the other for a given retention volume will be independent of p_0; consequently it will be possible to work at a low ratio of p_1/p_0 even with a very long column.

Test of general theory

Test of relationship between pressure, flow rate and retention volume. An experimental test of the theory given above was carried out by the procedure which is described later in the paper. A 4 ft. silicone-stearic acid column was set up and a series of separations of acetic, propionic, *n*- and *iso*-butyric acids carried out at four different pressures. The flow rates were measured in each case and from the graphs the retention volume of each acid band was measured.

The theoretical ratio of flow rates is given by (1):

$$\frac{F_1}{F_2}=\frac{(p_1/p_0)^2-1}{(p_2/p_0)^2-1}.$$

In Table 1 the theoretical ratios of the flow rates are compared with those experimentally determined; agreement is within expected error.

Table 1. *Comparison of calculated with experimentally determined flow rates in a gas-liquid partition chromatogram operated at different pressure ratios*

(The rates in runs *a–d* (Table 2) are compared. For basis of calculation see text.)

	Flow-rate ratios	
	Calc.	Found
a/*b*	1·44	1·46
a/*c*	1·92	1·96
a/*d*	3·36	3·21

From Eqn. 3 it can be seen that as the ratio of the pressures at the two ends of the column tends to unity the factor $\frac{(p_1/p_0)^3-1}{(p_1/p_0)^2-1}$ tends to 3/2. At this limiting pressure ratio the retention volumes of the bands tend to a limit V_R^0. In Fig. 1 the values of

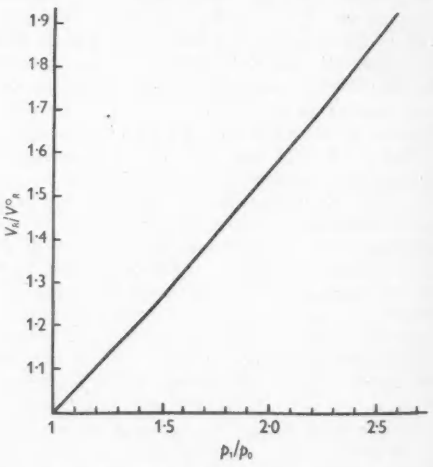

Fig. 1. The relationship between the pressure ratio across the column and the ratio between the observed retention volume and the retention volume at zero pressure difference across the column.

p_1/p_0 are plotted against the value of the ratio V_R/V_R^0; by means of this graph the experimental value of V_R can be corrected to V_R^0. In Table 2 these values of V_R^0 are shown for four experiments carried out at different values of p_1/p_0. It can be

Table 2. *Correction of retention volumes to their limiting values (V_R^0) for runs at different pressure ratios*

Run	Pressure of N_2 applied to column (cm. Hg)	p_1 (N_2 pressure plus atmospheric pressure, p_0, in cm. Hg)	p_1/p_0	V_R^0 (ml.) Acetic acid	Propionic acid	*iso*Butyric acid	*n*-Butyric acid
a	76·0	150·91	2·014	203	466	758	952
b	57·1	132·0	1·762	193	457	749	932
c	45·7	120·61	1·647	194	455	740	922
d	28·9	103·81	1·385	193	464	763	954

seen that the figures agree within 5%, a fairly satisfactory confirmation of the theory, since the assumption is made that the column is uniformly packed.

Derivation of column efficiency. Two methods of calculating column efficiency from the experimental curves are given in order to allow comparison with one another and with data from liquid-liquid columns.

Method 1

Let there be r plates in the column.

Assume that all the material (M equivalents) is put on in the first plate. Then the quantity occurring in the last plate at the maximum concentration is $M/\sqrt{(2\pi r)}$ equivalents (see Martin & Synge, 1941).

Let q be the fraction of the original quantity occurring in any plate, and let the time taken for the centre of the zone to reach the last plate be t min.; then the time taken for contents of last plate to escape $= t/r$ min.

Then $Mq_{max.} = M/\sqrt{(2\pi r)}$ equivalents and $Mq_{max.}\, r/t =$ max. rate of escape = max. slope of the experimental curve $= S$ equiv./min.

Then $$S = Mr/t\,\sqrt{(2\pi r)} \text{ equiv./min.}$$

Therefore $$r = 2\pi S^2 t^2/M^2.$$

Thus by measuring the maximum slope of the effluent concentration curve, the time to arrive at the maximum slope and the total amount of the material in the zone, the plate number of the column and hence the height equivalent to a theoretical plate (H.E.T.P.) can be obtained. H.E.T.P. is defined for the chromatogram by Martin & Synge (1941).

Method 2

The area between ordinates one standard deviation on either side of the peak of an error curve is 68·3% of the total. From the theory given by Martin & Synge (1941) the shape of the chromatographic zone should approximate to an error curve and 68·3% of the material should be included between plates $\pm\sqrt{r}$, where r is the total plate number.

The ratio of time taken for the centre of the zone to emerge (t) to the time taken for the middle 68·3% of the zone (τ) is equal to $r/2\sqrt{r}$.

Hence $$r = 4t^2/\tau^2.$$

A comparison of figures obtained by the two methods for the same column gives fair agreement (Table 3), the agreement increasing with the plate number.

Table 3. *Comparison of column efficiencies obtained by two independent methods*

(For methods 1 and 2 see text.)

Flow rate (ml./min.)	Plate number calculated by Method 1	Method 2
35	550	400
18·2	718	600
10·3	765	730

FACTORS INFLUENCING THE DEGREE OF SEPARATION

Column efficiency

Since diffusion in the gas is so much more rapid than in the liquid, diffusion in the liquid phase will normally be the factor limiting efficiency. At very slow rates of flow lengthways diffusion in the gas along the tube becomes relatively important, and then the efficiency is no longer increased by reducing the rate of flow. Table 4 shows the change of efficiency with flow rates above this limiting value.

Table 4. *Variation of column efficiency with flow rate*

Flow rate (ml./min.)	Efficiency (calc. by method 1) (no. of plates)
35	580
24	540
18·2	720
10	740

The H.E.T.P. shows slight variations from column to column due to differences in the uniformity of packing and of dispersion of the liquid phase. The plate number of any column is proportional to its length, the 4 ft. columns described later having plate numbers varying from 700 to 1200 plates and an 11 ft. column had a plate number of 2000, which might have been greater had it not been for the increased amount of dead space at the bends necessitated by the method of construction.

When diffusion in the liquid phase is the limiting factor, the H.E.T.P. is inversely proportional to the diffusion constant and hence an increase in temperature will lead to an increase in efficiency as the diffusion constant increases. An experimental test showed an efficiency of 365 plates at 65° and an

efficiency of 720 plates at 100° with the same column. This would suggest that in many cases the efficiency of liquid-liquid partition columns could be increased by raising the temperature (cf. Mayer & Tompkins, 1947).

Some variation of efficiency with the nature of the liquid phase will occur because the diffusion constant of the solute in the liquid phase will usually be inversely proportional to the viscosity of the liquid; hence fluids of low viscosity should in general be used.

Drake (1949) has shown that increasing the diameter of a chromatographic column leads to some loss of uniformity of front, and his results could be roughly covered by the assumption that the front made a given angle to the axis of the column irrespective of the column diameter. Since, however, with the gas-liquid columns the ratio of length to diameter is very large, moderate increases in diameter should be without measurable effect on efficiency; in fact one 4 ft. column of diameter 1·2 cm. had an efficiency of 850 plates, while with those of 4 mm. diameter the efficiency ranged from 700 to 1200 plates (at 100°).

Change of R_F

Inasmuch as in a homologous series the higher members will have larger latent heats of vaporization, their change of vapour pressure with temperature will be larger than with the lower members, so that the factor of separation will decrease as the temperature increases. This point is demonstrated in Table 5.

Table 5. *Variation of relative R_F values with temperature*

Ratio of retention volumes	Temperature of column 65°	100°	137°
$\frac{V_R \text{ propionic}}{V_R \text{ acetic}}$	2·9	2·3	2·1
$\frac{V_R \text{ } n\text{-butyric}}{V_R \text{ propionic}}$	2·5	2·0	1·8
$\frac{V_R \text{ } n\text{-butyric}}{V_R \text{ } iso\text{butyric}}$	1·34	1·27	1·20

Relative R_F values can be altered by changing the nature of the liquid phase. For example, replacement of the stearic acid in the columns described later by ricinoleic acid causes a disappearance of the α-methylbutyric-*iso*valeric acid and the αα-dimethylpropionic-*n*-butyric acid separations without markedly impairing the separation of *n*- and *iso*-acids. Incorporation of orthophosphoric or pyrophosphoric acids in the liquid phase of the silicone-stearic acid columns has a similar effect, but also decreases all R_F values, at the same time decreasing the ratio between them. This effect can no doubt be attributed to an increase in internal pressure of the liquid phase; a similar but more pronounced effect has been noticed in the separation of amines in the comparison of columns with liquid phases of hendecanol or glycerol. In this case the difference in pressure is so great that an inversion of order with length of chain is observable (James, Martin & Smith, 1952).

APPARATUS

Preparation of the column

Kieselguhr (Celite 545, Johns Manville Co. Ltd., obtainable from Imperial Chemical Industries Ltd.) is size-graded by repeated suspension in water in a beaker 18 cm. high and rejection of all fine material that does not settle out in less than 3 min. The resultant coarse-grade material is heated in a muffle for 3 hr. at 300° and then washed with conc. HCl to remove iron and basic impurities. After repeated washing with water to remove acid the kieselguhr is oven-dried at 145°. The liquid phase consisting of DC 550 silicone (Albright & Wilson Ltd., Oldbury, Birmingham), containing 10% (w/w) stearic acid, is added to the kieselguhr (0·5 g. of liquid phase to 1 g. kieselguhr) and the mixture is well stirred.

4 ft. column. A 5 ft. length of 4 mm. internal diameter glass tube is drawn down at one end to form a short length of thick-walled capillary (*F*, Fig. 2). A small wad of 'Fibreglass' yarn (900/1/41 obtainable from Fibreglass Ltd., Piccadilly, London) is pushed down the tube up to the capillary to prevent it being blocked by the kieselguhr. A small funnel is attached to the tube by a short length of pressure tubing and is then half filled with the kieselguhr mixture: with the column approximately vertical it is pressed against the rotating shaft of an electric motor (7000 rev./min.), the shaft having a flat machined on it. When all the kieselguhr has run into the column, the tube is pressed against the motor shaft until the kieselguhr packs down tightly, and this is assisted by a 'Telcothene' plunger attached to a length of steel wire. This process is repeated until 4 ft. of the column is

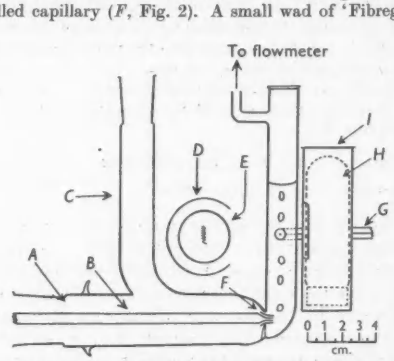

Fig. 2. Side view of titration-cell unit. *A*, vapour jacket; *B*, chromatogram; *C*, air-condenser socket; *D*, lamp house; *E*, lamp; *F*, rubber gasket on capillary; *G*, capillary lead from burette; *H*, photoelectric cell; *I*, photoelectric-cell housing with adjustable window.

packed and a second wad of 'Fibreglass' is pushed down on to the packing. A short piece of cycle-valve tubing is pushed on to the capillary end of the tube and acts as a gasket to prevent leakage from the titration cell into the vapour jacket.

11 *ft. column.* This column is constructed of three lengths of packed tubing joined by hairpin bends of thick-walled capillary. In this way the whole column can be accommodated within the same vapour jacket as the 4 ft. column. This principle can be used for very long columns if a wide vapour jacket is used.

the bubble past the graduations is timed with a stopwatch. An error of less than 1 % is possible over a wide range of flow rates.

The manostat by-passes to the atmosphere any excess gas coming from the nitrogen cylinder through a filter paper fixed with sealing wax to the bottom of the vent tube, when the mercury level is depressed beneath it. The pressure at which this occurs can be varied by adjustment of the level of the mercury reservoir. 'Hunting' is prevented by tightening the screwclip until critical damping occurs.

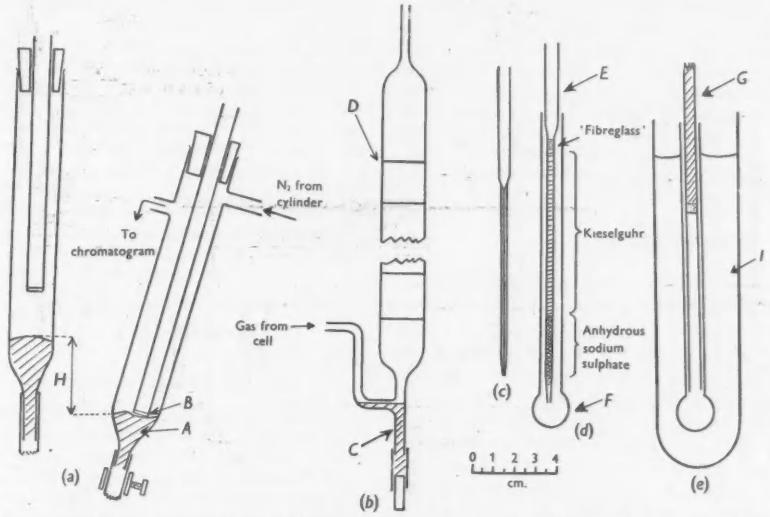

Fig. 3. (*a*) Manostat. *A*, mercury; *B*, vent tube fitted with filter-paper disk; *H*, effective nitrogen head. (*b*) Flowmeter. *C*, ammonium stearate solution; *D*, 10 ml. graduations. (*c*) Micropipette. (*d*) Extraction of free fatty acids into ether. *E*, partition column; *F*, test tube. (*e*) Displacement of fatty acids on to a gas-phase column. *G*, gas-phase chromatogram tube; *I*, heating bath.

Vapour jacket and titration cell

The vapour jacket, a horizontal 4 ft. 1 in. diam. glass tube with a ground-glass joint fitting the titration-cell unit (Fig. 2), is attached at the other end to the boiler by a ground-glass joint on a side arm. The chromatogram is supported in the jacket by a rubber bung and in the titration cell unit by the valve-tubing gasket (Fig. 2). The gasket, being rubber, limits the choice of liquid in the boiler; methanol (b.p. 65·4°), water (b.p. 100°), ethylene glycol monoethyl ether ('cellosolve') (b.p. 137°) and ethylene glycol (b.p. 200°) are suitable liquids. An air condenser is used for the high-boiling liquids. Phenol red was found to be the most suitable indicator in the titration cell for both visual (0·001 %) and automatic (0·01 % (w/v) aqueous solution) control of the titration.

Flowmeter and manostat (Figs. 3*b*, *a*)

These are both of conventional design.

A strong solution of ammonium oleate is used in the flowmeter; when the rubber tube is squeezed the solution rises into the side tube so that a bubble is formed. The passage of

Recording burette (Fig. 4)

The burette consists of a horizontal glass tube with a 'Telcothene' (Telegraph Construction and Maintenance Co. Ltd., Telcon Works, Greenwich, London, S.E. 10) gland (*I*) at one end through which passes an 0·125 in. diam. stainless-steel rod (*E*). A swelling on the burette tube where it passes through the end plate of the apparatus is insulated with a short length of rubber tube and is held in position by a small clamp (*J*), in this way providing a firm, but slightly flexible, attachment. A similar device allows pressure to be applied to the polythene gland without danger of cracking the tube. The other end of the burette is connected to two valves (*K*) consisting of a short length of pressure tubing whose bore is blocked by a short plug of glass rod; the valves may be opened by tightening a screwclip on to the plug. These valves have been found to be more reliable than glass taps. One valve leads to a reservoir fitted with a guard tube of soda lime, and the other to the titration cell through a length of capillary tube.

The free end of the stainless steel rod is attached to a carriage (*H*) sliding on two horizontal 0·5 in. steel rods (*C*). A lead screw (*D*, 1 mm. pitch, O.B.A. thread) between these

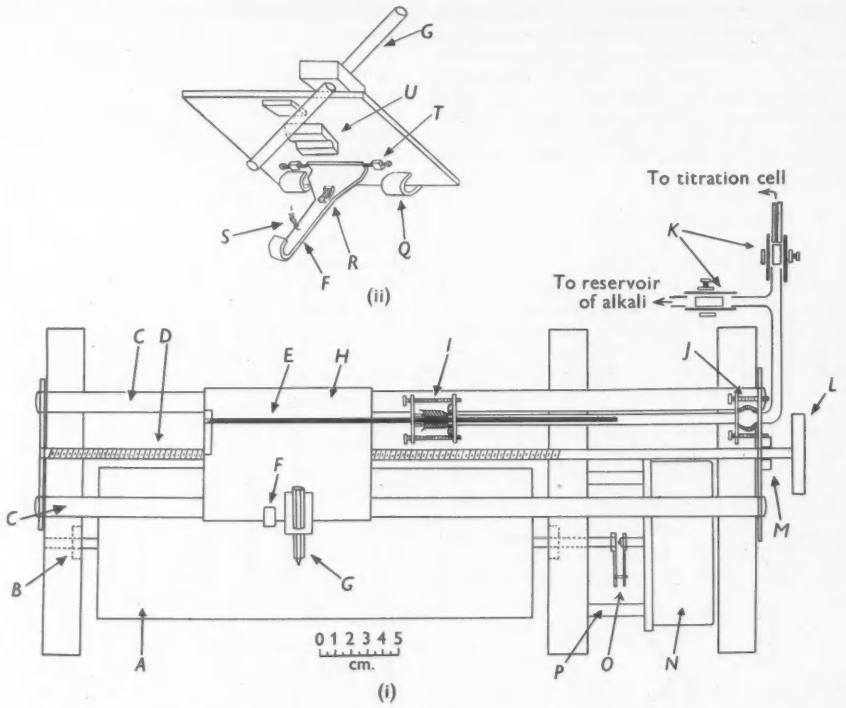

Fig. 4. (i) Plan view of automatic burette: *A*, rotating drum; *B*, drum-shaft bearing; *C*, steel rods; *D*, lead screw; *E*, stainless-steel rod; *F*, arm to disengage lead screw; *G*, pen; *H*, carriage; *I*, 'Telcothene' gland; *J*, burette-tube clamp; *K*, valves; *L*, pulley; *M*, lead-screw bearing; *N*, electric-clock movement; *O*, drum drive coupling (only one fork is shown); *P*, clock spacers. (ii) Underside view of carriage (flat springs holding front runner against rods are not shown): *F*, arm bearing half nut; *G*, pen holder; *Q*, carriage runners; *R*, half nut; *S*, spring to hold half nut against lead screw (shown disengaged from the carriage); *T*, pivots of half-nut support; *U*, 'Tufnol' (Tufnol Ltd., Perry Barr, Birmingham) support for lead screw.

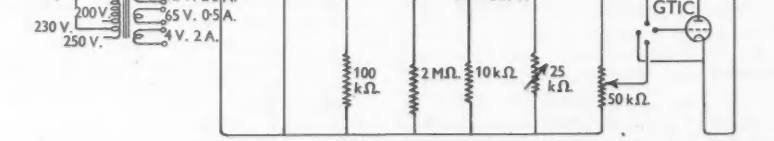

Fig. 5. Circuit of photoelectric control unit.

bars engages with a half nut on a plate pivoted at the back of the carriage (*F*, ii). By the depression of this plate against the action of a spring (*S*, ii) the half nut can be disengaged from the screw to allow rapid filling of the burette. A drum rotates on an axle driven by an electric-clock movement (*N*, Movement 55558/55, 10W. rating, English Clock Systems Ltd., 179 Great Portland Street, London, W. 1) parallel to the lead screw, and the position of the carriage at any time is recorded by a ball-point pen (*G*). The pen is held in a weighted tube (22 g.) which slides freely in a tube (*G*, ii) attached to the carriage, which makes an angle of 70° to the tangent at the point of contact with the drum. Round the drum, whose circumference is 30 cm., a piece of cm.-ruled graph paper is retained by means of two endless bands of 0·125 in. helical spring which can be readily rolled off the paper to the ends of the drum in order to change the paper. The drum can be rotated either once per hr. or once per 12 hr. by adjusting the position of a pin on a crank at the end of the drum shaft to engage either with a fork on the minute shaft of the clock (*O*) or with another fork on the hour shaft which rotates outside the minute shaft. In the diagram only the inner fork is shown.

The recording burette can be used manually by a handle on the lead screw. It is a very great convenience to use such apparatus instead of other types of burette and a stop-watch. Where the apparatus is expected to be in constant commission the photoelectric control unit will quickly repay the labour involved in its construction.

Photoelectric control unit

The circuit used is shown in Fig. 5. The tube GTIC is a gas-filled triode. The photoelectric cell (V.A. 26T Cinema-Television Ltd.) is predominantly green sensitive and consequently no filter is necessary when used with phenol red or methyl red as indicator. The photoelectric cell is provided with an adjustable window so that the area of the titration cell scanned can be varied. The lamp used to illuminate the cell is a 12V. (20W.) car bulb. The lamp and photocell are appropriately housed so that a minimum of daylight falls on to the cell, but so that visual observation of the titration is possible. The motor driving the lead screw is fitted with a reduction gear so that the maximum speed of the lead screw is of the order 30 rev./min.; a series rheostat is inserted in the motor circuit so that critical damping of the titration can be obtained.

APPLICATION TO THE SEPARATION OF THE VOLATILE FATTY ACIDS

Experimental procedure

The chromatogram supported in the vapour jacket is maintained at a temperature such that the saturated vapour pressures of the acids to be separated lie between 10 and 1000 mm. Hg. The temperatures used are 100° (boiling water) for acids from formic to valeric acid and 137° (boiling cellosolve) for acids from valeric to dodecanoic acid. The mixture of acids free from water is applied by means of a micropipette to the 'Fibreglass' plug in contact with the column packing, the N_2 supply from the manostat is attached, the electric clock driving the recording drum is started, and 8 ml. of aqueous phenol red solution are run into the titration cell. If very low rates of flow are used additional stirring is provided by a stream of N_2 through a stainless-steel capillary tube. The photoelectric control unit is then switched on. The acids emerge in the gas phase and are absorbed in the water in the titration cell where they are continuously titrated, and simultaneously the titration curve is automatically plotted. The curve obtained is the integral of the conventional chromatogram peak (cf. Moyle, Baldwin & Scarisbrick, 1948); the height of each step gives directly the amount of acid, each cm. corresponding to 3 μg. equiv. throughout these experiments, 0·038N-NaOH being used in the burette. This method of detection of the acids by titration in an aqueous medium limits the chain length of the acids that can be separated; with acids of chain length greater than C_{12} the columns would have to be run at a temperature of 180–200° in order to obtain a reasonable retention volume. The maximum possible temperature of the titration cell is 100° (using water) and with a temperature drop of 80–100° the acids will condense inside the capillary at the end of the column and so be prevented from reaching the titration liquid. Titration in non-aqueous media has not in our experience proved satisfactory.

RESULTS

The theory given above assumes that the partial pressures of substances to be separated are negligible; this may not always be so in practice. When they are not negligible the shapes of the zones are distorted because the velocity of gas is appreciably higher in the regions where the partial pressure is high, and this results in regions of high concentration tending to overtake regions of low concentration so that the zone will have a sharp front and a correspondingly longer tail. However, in all the examples quoted below, the partial vapour pressure (estimated from the maximum slope of the recorded step) can be neglected as it does not exceed 5 mm. Hg.

When the solutes obey Henry's law the partition coefficient between the two phases will be independent of concentration and the titration curve obtained will have a symmetrical sigmoid shape. Complete resolution of two zones will result in a plateau between the two curves. Any deviation from Henry's law will cause a deviation, of step shape, from the expected sigmoid curve.

Curve *A* (Fig. 6) shows the curve obtained in an attempted separation of propionic, *iso*butyric and *n*-butyric, and *iso*- and *n*-valeric acids on a column at 100°, having as liquid phase the high boiling-point silicone DC 550. The breaks in the curve indicate the ends of the different zones. The extended front and sharp tail of the zones of the higher acids is presumably due to dimerization in the liquid phase causing regions of low acid concentration (relatively high monomer to dimer ratio) to move more rapidly down the column than regions of high acid concentration (relatively low monomer to dimer ratio). This is because the partial pressure of the acid in the gas is nearly proportional to the concentration of the monomer in the liquid phase.

Incorporation of a long-chain fatty acid of low vapour pressure in the liquid phase will decrease the concentration of monomer by association to such a value that the deviation from ideal zone shape caused by dimerization will be much less. Curve *B* (Fig. 6) shows the result obtained when stearic acid (10 % w/w) is incorporated in the silicone liquid phase. The acids are now completely resolved and the *iso*valeric acid zone is shown to consist of two components, the second of which proved to be α-methylbutyric acid ('active' valeric acid). This result was obtained with all commercial samples of '*iso*valeric' acid tested (cf. Conrad & Bischoff, 1880; Kahlbaum, 1894). The increased retention volumes are a measure of the effectiveness of the volatile acid-stearic acid association and indicate

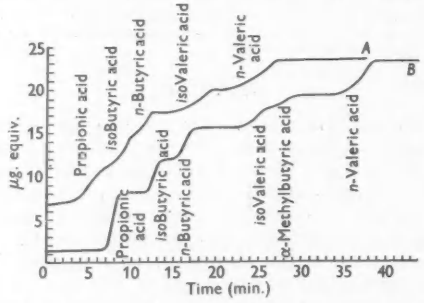

Fig. 6. The separation of propionic, *iso*butyric, *n*-butyric, commercial '*iso*valeric' and *n*-valeric acids. *A*, column length, 4 ft.; liquid phase, DC550 silicone; nitrogen pressure, 30 cm. Hg.; flow rate 23 ml./min.; column temp., 100°. Incomplete resolution of all the acids. *B*, column length, 4 ft.; liquid phase, stearic acid (10% w/w) in DC550 silicone; nitrogen pressure, 46 cm. Hg; flow rate, 45 ml./min.; column temp., 100°. The '*iso*-valeric' band has been almost completely resolved into two components.

a diminution of self-dimerization. Most commercial samples of stearic acid contain appreciable amounts of the lower homologues so that a freshly prepared column loses acid continuously to the titration cell. In so far as the rate of loss of acid is constant, it does not give rise to error in the estimation, but the step has to be measured against a sloping base-line. By running the column at a temperature 50° higher than the normal working temperature for a few hours the volatile impurities can be effectively removed. With pure stearic acid the loss of acid is very small, and in addition the liquid in the titration cell rapidly becomes saturated and thereafter no further titration of the stearic acid entering the cell occurs.

The lower curve (*A*) of Fig. 7 shows the separation of acetic, propionic, *iso*butyric and *n*-butyric acids on a similar silicone-stearic acid column at a smaller flow rate. The upper curve (*B*) is obtained by graphical differentiation of the lower curve and shows a change of zone shape along the series. It is clear that the zones still deviate from the theoretical

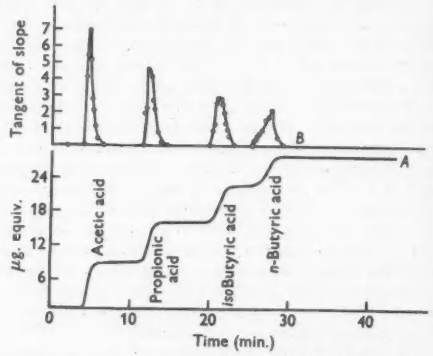

Fig. 7. The separation of acetic, propionic, *iso*butyric and *n*-butyric acids. Column length, 4 ft.; liquid phase, stearic acid (10% w/w) in DC550 silicone; nitrogen pressure, 48 cm. Hg; flow rate, 33 cm./min.; column temp., 100°. *A*, experimental curve. *B*, differential of experimental curve.

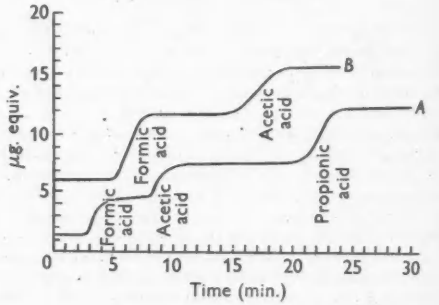

Fig. 8. *A*, the separation of formic, acetic and propionic acids showing tailing of formic acid band. Column length, 4 ft.; liquid phase, stearic acid (10% w/w) in DC550 silicone; nitrogen pressure, 25 cm. Hg; flow rate, 15 ml./min.; temp., 100°. *B*, the separation of formic and acetic acids, showing nearly symmetrical formic acid band. Column length, 4 ft.; liquid phase, stearic acid (10% w/w), orthophosphoric acid (10% w/w) in DC550 silicone; nitrogen pressure, 70 cm. Hg; flow rate, 50 ml./min.; temp., 100°.

The lower curve (*A*) of Fig. 7 shows the separation of acetic, propionic, *iso*butyric and *n*-butyric acids on a similar silicone-stearic acid column at a smaller flow rate. The upper curve (*B*) is obtained by graphical differentiation of the lower curve and shows a change of zone shape along the series. It is clear that the zones still deviate from the theoretical shape. The acetic acid and propionic acid zones have sharper fronts than tails, the *iso*butyric acid zone is almost symmetrical and the *n*-butyric acid zone has a sharper tail than front. The sharp fronts of the acetic and propionic acid zones are to be attributed to adsorption on the kieselguhr since when the acids were run on a column containing no liquid phase

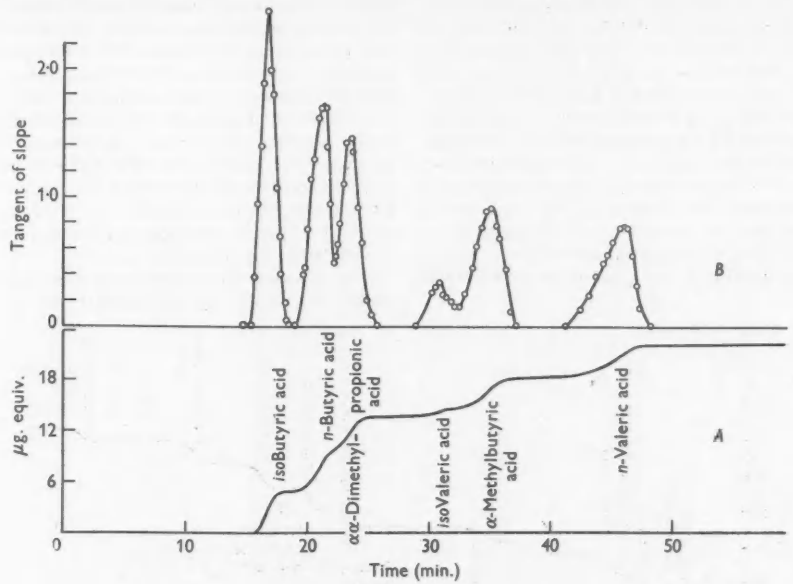

Fig. 9. The separation of the isomers of valeric acid from *n*-butyric and *iso*butyric acids, showing incomplete resolution of αα-dimethylpropionic and *n*-butyric acids and of *iso*valeric and α-methylbutyric acids. *A*, experimental curve. *B*, differential of experimental curve. Column length, 4 ft.; liquid phase, stearic acid (10% w/w) in DC550 silicone; nitrogen pressure, 44 cm. Hg; flow rate, 43 ml./min.; temp., 100°.

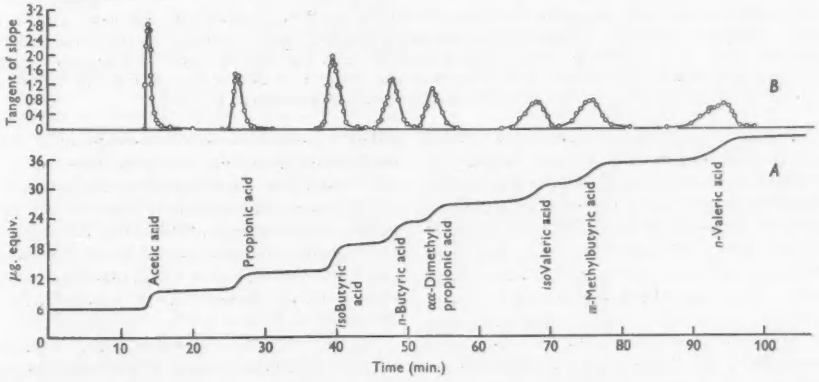

Fig. 10. The separation of acetic, propionic, *n*-butyric and *iso*butyric acids and the isomers of valeric acid, showing the complete resolution of all bands, and change in band shape in ascending the series. *A*, experimental curve. *B*, differential of experimental curve. Column length, 11 ft.; liquid phase, stearic acid (10% w/w) in DC550 silicone; nitrogen pressure, 74 cm. Hg; flow rate, 18·2 ml./min.; temp., 137°.

they showed a non-linear adsorption isotherm with the tail of the acetic zone delayed by some 50 ml. at 100°. This delay increased with the chain length of the fatty acid. In the case of acetic and propionic acids the distortion caused by the adsorption is sufficient to mask completely the effect of the dimerization which has doubtless occurred. With the *iso*butyric acid the two effects are in approximate balance and a nearly symmetrical zone results; with *n*-butyric and higher acids the dominant effect is that of dimerization. Increasing the temperature appears to have a greater effect on dimerization than adsorption, since the symmetrical zone is then given by an acid higher than *iso*butyric acid (see also Fig. 10).

a 4 ft. silicone-stearic acid column at 100°. The upper differential curve (obtained as described earlier) shows that complete resolution of *n*-butyric and $\alpha\alpha$-dimethylpropionic acids and *iso*valeric and α-methylbutyric acids is not attained. To improve this separation a longer column was constructed. Curves *A* and *B* (Fig. 10) show the separation of acetic, propionic, *iso*butyric, *n*-butyric, $\alpha\alpha$-dimethylpropionic, *iso*valeric, α-methylbutyric and *n*-valeric acids on an 11 ft. silicone-stearic acid column at 137°. Complete resolution of all the acids is obtained. The marked change in zone shape with molecular weight is well shown in this series.

Fig. 11 shows the separation of acetic, propionic, *iso*butyric, *n*-butyric, commercial '*iso*valeric', *n*-

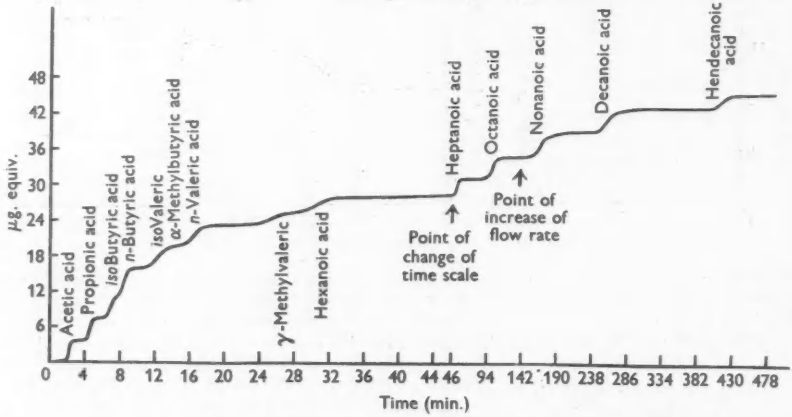

Fig. 11. The separation of acetic, propionic, *n*-butyric, *iso*butyric, *n*-valeric, commercial '*iso*valeric', γ-methylvaleric, hexanoic, heptanoic, octanoic, nonanoic, decanoic and hendecanoic acids. Column length, 4 ft.; liquid phase, stearic acid (10% w/w) in DC550 silicone; nitrogen pressure, 47 cm. Hg; flow rate, 18·2 ml./min.; temp., 137°. At the time indicated by the first arrow the time scale was changed to 24 min./cm. At the time indicated by the second arrow the pressure was increased to 76·5 cm. Hg and the flow rate to 35 ml./min.

Curve *A* (Fig. 8) shows the separation of formic, acetic and propionic acids on a silicone-stearic acid column. The formic acid zone shows marked tailing and extends backwards into the acetic acid zone so that complete resolution is not obtained. Curve *B* shows the separation of formic and acetic acids when orthophosphoric acid is incorporated in the column liquid phase. The shape of the formic acid zone now approximates to that of the acetic acid zone and indicates that the phosphoric acid has effectively suppressed adsorption. The greater retention volume for each zone also indicates greater association in the liquid phase. A similar effect on tailing is produced by washing the kieselguhr with dilute aqueous phosphoric acid (1% v/v) before the final drying.

Curve *A* (Fig. 9) shows the separation of *iso*-butyric, *n*-butyric, $\alpha\alpha$-dimethylpropionic, *iso*-valeric, α-methylbutyric and *n*-valeric acids on valeric, γ-methylvaleric ('*iso*caproic'), hexanoic, heptanoic, octanoic, nonanoic, decanoic and hendecanoic acids. Dodecanoic acid is not shown as it takes a longer time to emerge than can conveniently be shown on the graph (817 min.). The rate of flow is too high for complete resolution of the lower acids, but it can be seen that a rough quantitative analysis of *n*- and *iso*-acids from C_2 to C_6 can be carried out in 36 min.

All the columns used are highly reproducible; V_R^0 even at different rates of flow varies by less than 5% for a given acid (Table 2) and even the most difficultly separable acids with less than five carbon atoms, namely, $\alpha\alpha$-dimethylpropionic and *n*-butyric acids, have retention volumes differing by 15% (Table 6). It is thus possible to identify any of these acids by retention volume alone. No marked fall in efficiency with repeated use takes place, the factor

limiting the life being breakage and not deterioration. Recoveries are quantitative within the error of the titration and no indication of incomplete absorption in the titration cell has been found. We have not striven to reduce the errors below a few per cent, though we are of the opinion that the method is essentially a highly accurate one.

Table 6. *Retention volumes of the fatty acids relative to that of* n-*butyric acid on silicone-stearic acid columns*

Acid	B.p. (°)	Column temp. 100°	Column temp. 137°
Formic	107·7	0·076	—
Acetic	118·1	0·20	0·26
Propionic	141·1	0·47	0·54
*iso*Butyric	154·4	0·77	0·81
n-Butyric	163·5	1·0	1·0
αα-Dimethylpropionic	163·8	1·15	—
*iso*Valeric	176·7	1·51	1·48
α-Methylbutyric	177	1·70	—
n-Valeric	187	2·17	1·91
γ-Methylvaleric	199·1	—	2·94
Hexanoic	205	—	3·58
Heptanoic	223·5	—	6·55
Octanoic	237·5	—	12·0
Nonanoic	254	—	22·0
Decanoic	268–70	—	40·5
Hendecanoic	225*	—	72·8
Dodecanoic	225†	—	138·5

* 100 mm. Hg. † 40 mm. Hg.

A steady increase of retention volume with chain length is found to occur with the straight-chain acids, the retention volume increasing by a factor approx. 1·9 for each increase of chain length of one CH_2 group, formic acid showing the maximum deviation. Fig. 12 shows a plot of log V_R^0 against chain length. It can be seen that the curve joining the *iso* acids runs parallel to that joining the normal acids as would be expected.

The maximum permissible loading of the 4 mm. columns is approximately 1 mg. of each of the lower acids, but if the cross-section area of the column be increased the loading can be increased in the same ratio. The minimum amount of an acid detectable (using 0·038 N-base in the burette) is 0·3 μg. equiv., i.e. 0·02 mg. for acetic acid. As explained earlier, this lower level is determined only by the method of detection used.

The determination of fatty acids occurring as their sodium salts

In most biological experiments the volatile fatty acids are isolated as their sodium salts, and a simple method of obtaining the acids free from water is necessary before they can be applied to the column. The presence of even a small percentage of water in the fatty acid sample is sufficient to upset the separation, presumably owing to azeotrope formation.

The method adopted consists in drawing up a small sample of an aqueous solution of the sodium salts (30 μl. of an approx. 2 N solution) into a micropipette (Fig. 3*c*) that has been pretreated with a silicone (by washing with a 1 % (w/v) solution of DC 1107 in chloroform followed by heating at 140° for 30 min.), to render it unwettable by water. A small bubble of air is drawn in and an aqueous

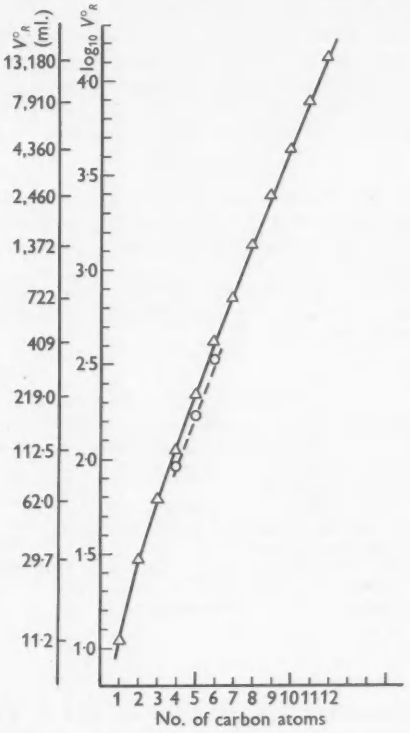

Fig. 12. The relationship between the retention volume at zero pressure difference (V_R^0) and the number of carbon atoms in the lower fatty acids. △, *n*-Acids; ○, *iso*-acids. Data are for a 4 ft. silicone-stearic acid column at 137° (cf. Fig. 11).

solution of orthophosphoric acid (60 % w/v) is drawn up and the two solutions mixed in the wide part of the pipette. The acidified mixture is then run on to the top of a micro-partition column (Fig. 3*d*), the lower part of which is packed with anhydrous sodium sulphate and the rest with kieselguhr (Celite 545). The aqueous solution of the acids is taken up by the kieselguhr. Ether (0·6 ml.) is then run through the micropipette on to the column and passed through the column under pressure. The eluate is collected in a long narrow test tube

(Fig. 3*d*), whose diameter is slightly greater than that of the chromatogram tube. When all the ether has been collected the chromatogram tube is removed from its vapour jacket and inserted in the test tube so that the end of the column is just above the surface of the ether. The test tube is immersed in warm water and air is drawn through the column until all the ether has evaporated. The test tube is then immersed in a heating bath (100° for acids up to valeric, 150° for acids up to dodecanoic acid) and air is drawn through the column for 15 min. The acids move up into the column and condense there. At the end of this time the column is replaced in the vapour jacket, the nitrogen gas turned on and the separation carried out as already described.

SUMMARY

1. The theory of the partition column has been extended to cover a compressible mobile phase. Satisfactory agreement of experiment with theory has been found.

2. Two methods of calculating the height equivalent to a theoretical plate in such columns are derived and compared.

3. Factors influencing the degree of separation are discussed.

4. Gas-liquid partition columns are described for the separation of volatile fatty acids from formic acid to dodecanoic acid.

5. Complete resolution is obtained of all normal acids (and *iso* acids up to at least C_6) on a 4 ft. column and of all the isomers of valeric acid on an 11 ft. column.

6. A method is described for the micro-estimation of volatile fatty acids isolated as their sodium salts.

7. A recording burette is described which may be coupled to a photoelectric control circuit to render the analysis automatic.

We wish to thank Dr R. Scarisbrick for a gift of α-methylbutyric acid, Mr E. A. Piper and Mr C. C. F. Payne for the development and construction of the photoelectric control circuit, Mr C. W. Couling and Mr G. V. Arthur for the tracing of the experimental curves, and Mr C. Sutton and co-workers for the photographic copying of all diagrams.

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Quantitative Studies of the Avidity of Naturally Occurring Substances for Trace Metals

2. AMINO-ACIDS HAVING THREE IONIZING GROUPS

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The fact that glycine can successfully compete for copper ions against six times its weight of serum albumin (Klotz & Fiess, 1951) suggests that the metal-binding powers of the amino-acids are of a relative magnitude sufficient to play an important part in the metabolism of trace metals.

The constants governing equilibria between the ions of heavy metals and those amino-acids that have only two ionizing groups were presented in Part 1 (Albert, 1950). The present paper deals with the constants of amino-acids having three such groups. The only relevant constants in the literature are for aspartic acid with copper (Li & Doody, 1950) and magnesium (Greenwald, 1939) and for histidine

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with cobalt (Hearon, Burk & Schade, 1949) and copper, zinc, cobalt and manganese (Maley & Mellor, 1950).

It will be seen that the amino-acids dealt with here present more examples of individuality than those investigated in Part 1. Several of the acids possess avidity of a much higher order than has previously been encountered in this work.

METHODS

The substances investigated were all obtained from British Drug Houses Ltd., except the following: diaminobutyric and diaminopropionic acids (Drs T. S. G. Jones and S. Wilkinson of the Wellcome Research Laboratories, Beckenham, Kent) and benzoylglutamic acid (Dr A. L. Levy). Tyrosine, histidine and glutamic acid were of the grade 'microbiologically tested'. Tyrosine failed to pass the tests for purity described in Part 1 and was recrystallized from water until satisfactory. Cysteine hydrochloride was dried for 18 hr. in a vacuum over $CaCl_2$ at 20°; the other substances were dried in air at 115° for 1 hr.

Titrations. The ionization constants, reported in Table 3, were in general determined in water at 0·01 M (0·005 M for tyrosine, because of its sparing solubility). However, values of pK_a less than 2 or greater than 12 have been taken from the literature. The values for diaminopropionic acid, diaminobutyric acid and benzoylglutamic acid are reported here for the first time (the values below 2 were found at 0·1 M). The two constants of benzoylglutamic acid which lie close together were separated by the method of Noyes & Britton (Britton, 1942).

Titrations in the presence of metallic cations were carried out at 20°, in general as in Part 1, but solubility considerations necessitated solutions more dilute than the standard (0·01 M-amino-acid and 0·005 M-metallic salt) in the following cases: tyrosine (0·005 M), all metals; cysteine (0·0025 M), Zn^{++}; diaminopropionic acid (0·005 M), Zn^{++}, Fe^{++}; diaminobutyric acid (0·0025 M), Zn^{++} and Fe^{++}, (0·005 M) with Mn^{++}; lysine (0·0013 M), Fe^{++}; ornithine (0·0025 M), Fe^{++}; histidine (0·005 M), Zn^{++}; aspartic acid (0·005 M), Fe^{++}; glutamic acid (0·0025 M), Fe^{++}, Mn^{++}.

The amount of alkali used for each titration was 10 ml. of 0·1 N-KOH except for cysteine hydrochloride and lysine dihydrochloride where 15 ml. were required. Boiled-out water was used for all the cysteine and histidine titrations, but otherwise only when Co^{++}, Fe^{++} or Mn^{++} were used.

Symbols.

$$K' = \frac{[\text{the 1:1 complex}]}{[\text{free metallic ions}] \times [\text{free complex-forming species}]} \quad (a)$$

$$K'' = \frac{[\text{the 2:1 complex}]}{[\text{the 1:1 complex}] \times [\text{free complex-forming species}]} \quad (b)$$

By combining these two chemical equations or, which is the algebraic equivalent, multiplying (*a*) and (*b*), an overall stability constant (K_s) for the entire reaction is obtained; hence

$$K_s = \frac{[\text{the 2:1 complex}]}{[\text{free metallic ions}] \times [\text{free complex-forming species}]^2}$$

or, more succinctly,

$$K_s = K' . K''.$$

$\bar{n}$ The average number of molecules of complex-forming agent bound by one atom of metal.

[Sc] The concentration of the complex-forming species, which is $NH_2\text{-}CH_2\text{-}CO_2^-$ in the case of glycine.

[XSc°] The total concentration of the amino-acid, including all species, bound or free.

[M°] The total concentration of metal, bound or free.

$$\alpha = \frac{[H^+]}{K_a} + \frac{[H^+]^2}{K_a K_a'} + \frac{[H^+]^3}{K_a K_a' K_a''} + 1.$$

$$\beta = \frac{[H^+]}{K_a} + \frac{2\,[H^+]^2}{K_a K_a'} + \frac{3\,[H^+]^3}{K_a K_a' K_a''}.$$

K_a is the numerically largest ionization constant (e.g. $10^{-9\cdot20}$ for histidine), K_a' the second largest, K_a'' the third largest. When the pH is more than 1·4 units above pK_a', the second term in these equations can be neglected; when the pH is more than 1·4 units above pK_a'', the third term can be neglected.

Calculations. K_s, the overall stability constant, was calculated from (i)

$$\log K_s = -2 \log [\text{Sc}], \text{ when } \bar{n} = 1{\cdot}00, \quad (i)$$

and then compared with independent calculation from (ii), after the manner of Table 1. This precaution was found to be necessary to check the symmetry of the formation curves (symmetry being essential for the validity of (i)) and also to confirm that the 1:1 addition of molecules of amino-acid to each atom of the metal took precedence over the 2:1 addition

$$K_s = K' . K''. \quad (ii)$$

When K'' could not be determined, owing to insufficient solubility of the complex, K' alone is reported. K', K'' and $\bar{n}$ were calculated by equations (iii), (iv) and (v) respectively, as in Part 1.

$$K' = \bar{n}/(1 - \bar{n})\,[\text{Sc}], \quad (iii)$$

$$K'' = (\bar{n} - 1)/(2 - \bar{n})\,[\text{Sc}], \quad (iv)$$

$$\bar{n} = \frac{[\text{XSc}^\circ] - \alpha\,[\text{Sc}]}{[\text{M}^\circ]}. \quad (v)$$

[XSc°] was called [HSc°] in Part 1; the other symbols have the meaning previously given to them.

The derivations of (v) and (vi), which were omitted from Part 1, are given in the Appendix to this paper.

[Sc] was calculated from (vi) and (vii) respectively, depending on whether two or four hydrogen ions were eliminated from two molecules of the complex-forming substance (a test for the number of hydrogen ions is given on p. 692).

A worked example of the calculation of $\log K_s$ from (vi) appeared in Part 1. A worked example using (vii) forms Table 1 of the present work.

$$\log [\text{Sc}] = \log \{[\text{XSc}^\circ] - [\text{KOH}] - [H^+]\} - \log \beta, \quad (vi)$$

$$\log [\text{Sc}] = \log \{2[\text{XSc}^\circ] - [\text{KOH}] - [H^+]\} - \log \beta. \quad (vii)$$

Table 1. *Potentiometric titration of a mixture of αγ-diaminobutyric acid* (0·01 M) *and nickel nitrate* (0·005 M)

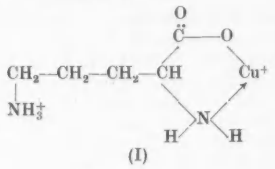
(These molarities are achieved in the middle of the titration where total volume is 50 ml. The pH readings are given in Table 2.)

0·1 N-KOH (ml.)	log [Sc]	n	log K'	log K''
0·5	$\bar{1}\bar{1}$·41	0·09	—	—
1·0	$\bar{1}\bar{1}$·98	0·20	9·42	—
1·5	$\bar{1}0$·28	0·30	9·34	—
2·0	$\bar{1}0$·55	0·40	9·27	—
2·5	$\bar{1}0$·74	0·50	9·26	—
3·0	$\bar{1}0$·97	0·60	9·20	—
3·5	$\bar{9}$·20	0·70	9·15	—
4·0	$\bar{9}$·38	0·80	9·22	—
4·5	$\bar{9}$·59	0·90	—	—
5·0	$\bar{9}$·81	1·00*	—	—
5·5	$\bar{8}$·04	1·10	—	—
6·0	$\bar{8}$·27	1·20	—	7·13
6·5	$\bar{8}$·47	1·30	—	7·15
7·0	$\bar{8}$·72	1·38	—	7·07
7·5	$\bar{8}$·91	1·48	—	7·05
8·0	$\bar{7}$·11	1·58	—	7·03
8·5	$\bar{7}$·25	1·68	—	7·08
9·0	$\bar{7}$·44	1·78	—	7·11
9·5	$\bar{7}$·73	1·88	—	—
log mean of antilogs			9·27	7·08†

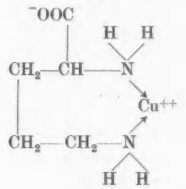
* Whence log K_s is 16·38 by equation (i).
† Whence log K_s is 16·35 by equation (ii).

RESULTS

The stability constants, where well established, are reported in Table 3. In order to apply the correct calculations, it was important to decide whether one or two hydrogen ions were being displaced per molecule of complex-forming agent. For example, ornithine hydrobromide might combine with copper as glycine does to give (I), resulting in the liberation of one hydrogen ion. Alternatively, the combination might involve both nitrogen atoms (as with ethylenediamine) to give (II), two hydrogen ions being liberated. When the pH is raised, each atom of copper is likely to combine with two molecules of amino-acid thus liberating, in all, two hydrogen ions if the complex is type (I) and four if it is type (II).



(I)



(II)

In order to decide whether a given combination would produce a complex of type (I) or (II), a comparison was made of the pH values obtained on titration with alkali in the presence and in the absence of the metal. For example, in Table 2, glycine shows evidence of end-point formation when 1 equivalent of alkali is added, because the pH obtained in the presence of the metal at 1·1 equiva-

Table 2. *Glycine and a homologous series of dibasic amino-acids. Comparison of* pH *values found on titration with alkali in absence and presence of metal cations*

(50 ml. of 0·01 M-amino-acid are titrated with 2 equivalents (10 ml.) of 0·1 N-KOH. The metal concentration is 0·005 M, where present.)

0·1 N-KOH	Glycine		Lysine			Ornithine			Diaminobutyric acid			Diaminopropionic acid		
(ml.)	Alone	+Cu++	Alone	+Cu++	+Ni++	Alone	+Cu++	+Ni++	Alone	+Cu++	+Ni++	Alone	+Cu++	+Ni++
0	6·12	3·18	6·25	3·23	4·03	6·02	3·12	4·38	5·77	2·90	4·18	4·26	2·56	3·43
0·5	8·88	3·37	8·04	3·47	5·71	7·63	3·33	5·55	7·30	3·10	5·05	5·68	2·68	3·92
1·0	9·30	3·56	8·55	3·72	6·23	8·00	3·58	5·95	7·63	3·32	5·35	6·05	2·84	4·27
1·5	9·48	3·83	8·82	4·00	6·52	8·26	3·83	6·23	7·91	3·56	5·51	6·32	3·01	4·50
2·0	9·72	4·10	9·05	4·28	6·82	8·45	4·11	6·53	8·05	3·85	5·66	6·51	3·20	4·68
2·5	9·86	4·46	9·20	4·62	7·07	8·63	4·42	6·75	8·23	4·11	5·77	6·69	3·44	4·84
3·0	10·05	4·86	9·37	4·98	7·37	8·81	4·78	6·99	8·41	4·43	5·90	6·88	3·67	4·97
3·5	10·20	5·30	9·53	5·37	7·66	8·99	5·15	7·22	8·59	4·81	6·03	7·07	3·90	5·10
4·0	10·40	5·74	9·66	5·89	7·96	9·17	5·61	7·50	8·83	5·15	6·14	7·29	4·12	5·22
4·5	10·63	6·50	9·80	6·52	8·35	9·38	6·24	7·72	9·06	5·52	6·26	7·61	4·34	5·34
5·0	10·77	8·41	9·94	8·53	8·80	9·59	7·54	7·95	9·35	5·81	6·39	8·13	4·54	5·45
5·5	10·97	10·65	10·01	9·34	9·30	9·95	8·43	8·17	9·66	6·06	6·53	8·62	4·70	5·57
6·0	11·20	11·04	10·08	9·70	9·64	10·16	8·86	8·40	9·88	6·32	6·67	8·93	4·86	5·68
6·5	11·38	11·24	10·38	9·94	9·90	10·34	9·14	8·61	10·10	6·57	6·80	9·18	5·04	5·82
7·0	11·53	Ppt.	10·52	10·12	10·09	10·53	9·39	8·83	10·24	6·86	6·96	9·35	5·20	5·94
7·5	—	—	10·66	10·27	10·26	10·66	9·62	9·04	10·40	7·25	7·10	9·53	5·36	6·05
8·0	—	—	10·80	10·43	10·39	10·81	9·85	9·26	10·57	7·72	7·25	9·71	5·53	6·20
8·5	—	—	10·91	10·57	10·52	10·96	10·08	9·53	10·72	8·06	7·39	9·86	5·74	6·38
9·0	—	—	11·09	10·72	10·66	11·08	10·31	9·80	10·86	8·34	7·58	10·04	6·00	6·60
9·5	—	—	11·18	10·85	10·78	11·22	10·54	10·12	11·02	8·77	7·90	10·24	6·46	7·02
10·0	—	—	11·28	10·98	10·90	11·33	10·76	10·52	11·18	9·64	8·49	10·52	9·42	10·05
10·5	—	—	—	—	—	—	—	—	11·32	10·70	10·69	—	—	—

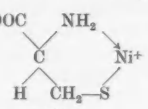
lents (5·5 ml.) is only 0·3 pH unit below that obtained in the absence of metal (nevertheless at 1·0 equivalent, the difference is 2·36 units). Diaminobutyric and diaminopropionic acids, on the other hand, show no tendency to form an end point at 1 equivalent, where the difference of 3 units of pH shows that hydrogen ions are still being liberated in great quantities. However, a typical end point is shown at 2 equivalents and hence it is concluded that four hydrogen atoms have been displaced (i.e. two/mol. of amino-acid). Thus, these acids utilize both nitrogen atoms in forming their complexes, which resemble (II). Their higher homologue lysine, however, is plainly type (I), whereas ornithine, which is intermediate in structure, forms mainly a type (I) copper complex but both types of nickel complex. More will be said about this homologous series in the discussion.

DISCUSSION

The presence of three ionizing groups introduces complexities which were not encountered in Part 1. The various acids will be discussed in the order in which they occur in Table 3.

Tyrosine. That the pK_a at 9·19 is indeed that of the α-amino-group follows from the work of Birch & Harris (1930) and Crammer & Neuberger (1943). The titrations proved to be quite straightforward, complexes of Type (I) being formed without the involvement of the phenolic group. Indeed the formation curves for the tyrosine-metal titrations are parallel to, and almost coincident with, those obtained for tryptophan (Part 1). Ferric ions appeared not to react with tyrosine. Davis & Waind (1950) found log $K_s = 1·48$ for calcium by a solubility method.

Cysteine gives by far the highest constants of any amino-acid. The complexes are of type (III), because three hydrogen ions were lost from each molecule of the hydrochloride. Of all the amino-acids discussed here, only cysteine was used in the form where a hydrogen ion is initially on the carboxyl group; as a result, the same sequence of pH readings was produced during the addition of the first equivalent of alkali regardless of whether a metal were present or not; differentiation occurred during the addition of the next equivalents of alkali, and hydrogen ions were still liberated copiously during the addition of the third equivalent.



(III)

The question whether the —COO^- group participates in ring formation of these type (III) complexes (or in the type (II) complexes of other amino-acids) is one that the present experimental method does not answer. In the case of ions such as Cu^{++}, Zn^{++}, Mn^{++} and Mg^{++}, which prefer to remain tetracovalent in solution, such participation is unlikely.

No stability constant for cupric cysteine could be found because of the reaction between cupric salts and cysteine hydrochloride to give a precipitate of cystine and cuprous cysteine (cf. Harris, 1922). In

Table 3. *Stability constants of the complexes of amino-acids and metallic cations* (20°)

(Reported as log K_s.)

Substance	Ionization constants α-COOH	α-NH_2	Other	Stability constants Cu^{++}	Ni^{++}	Zn^{++}	Co^{++}	Fe^{++}	Mn^{++}	Mg^{++}
Glycine (for comparison)	2·22	9·86	—	15·4	11·0	9·3	8·9	7·8	5·5	4
L-Tyrosine	2·20	9·19	10·43	15·0	10·1	9·1	8·1	7·1	(2·4)‡	(2)‡
DL-Cysteine HCl	1·96	8·36	10·28	†	19·3	18·2	†	(6·2)‡	(4·1)‡	(<4)‡
DL-αβ-Diaminopropionic acid HBr	1·23 (±0·03)	6·69 (±0·04)	9·50 (±0·04)	†	15·2	11·5	11·8	(5·0)‡	—	—
DL-αγ-Diaminobutyric acid HCl	1·85 (±0·06)	8·24 (±0·03)	10·44 (±0·06)	†	16·4	12·8	12·8	(5·7)‡	(4·2)‡	—
DL-Ornithine HBr	1·94	8·62	10·73	13·0	8·3	6·9	6·3	(5·0)‡	(<2)‡	—
L-Lysine 2HCl H_2O	2·18	9·18	10·72	13·7	8·8	7·5	6·8	(4·5)‡	(2·0)‡	—
L-Arginine HCl.H_2O	2·17	9·08	12·48	13·9	9·2	7·8	7·4	(3·2)‡	(2·0)‡	—
Ethylenediamine* (for comparison)	—	7·27	10·18	20·0	14·0	11·1	10·7	—	—	—
L-Histidine HCl.H_2O	1·82	9·20	6·08	†	15·9	12·0	†	9·3	(<4)‡	(<4)‡
Histamine 2HCl (for comparison)	—	9·82	6·03	16·2	11·7	8·7	8·7	—	—	—
L-Aspartic acid	2·08	9·98	3·94	†	12·7	10·4	10·7	8·5	(4·0)‡	—
L-Glutamic acid	2·19	9·92	4·33	†	10·3	8·5	8·1	(4·6)‡	(3·3)‡	—
L-Benzoylglutamic acid	3·49 (±0·05)	—	4·99 (±0·07)	(2)‡	—	—	—	—	—	—

* Values taken from Bjerrum (1950). Log K_s is here log $K'K''$, but this amine also forms stable 3:1 complexes with Ni^{++} and Co^{++}.

† See text, pp. 693–6.

‡ These are values for log K' only, because the insolubility of the complex interfered with the determination of K''.

view of the general instability of $Cu^{II}S$ bonds, more intracellular copper may be in the Cu^{I} condition than has been thought.

Cobalt and cysteine are known to give a variety of complexes which have been isolated and described by Schubert (1931, 1933). The simplest of these is the pale red-violet $K_2Co^{++}R_2$ (where R is $—SCH_2—CH.(NH_2)COO^-$). This is presumably a type (III) complex, although Schubert believed that the $—NH_2$ group was free in all cobalt-cysteine complexes. This substance in the presence of excess cysteine at a higher pH adds a further molecule of cysteine to give the blue-violet $K_4Co^{++}R_3$, where the co-ordination number of the (cobaltous) cobalt has been raised from 4 to 6. On exposure to air this complex becomes oxidized to the cobaltic state giving either a deep-red or a deep-green isomer of the formula $K_3Co^{+++}R_3$. Further exposure to air causes the formation of a brown complex, $(K_2Co^{+++}(OH)R_2)_2$, cystine being also formed.

Hitherto these complexes had not been investigated by potentiometric titration. This was undertaken with rigorous attention to the exclusion of oxygen. The solution was yellow before the titration began, but became increasingly green. As it is evident from Schubert's work that a minute amount of the green complex can cause $K_2Co^{++}R_2$ to appear green, it was assumed that very little oxidation had occurred. Hence constants were worked out for the titration, assuming the formation of a type (III) complex (in so far as there was no end point corresponding to one hydrogen ion per molecule). The formation curve was of good symmetry up to $\bar{n} = 1{\cdot}4$ (pH 7). From it the constants $\log K' = 8{\cdot}8$, $\log K'' = 7{\cdot}4$ (whence $\log K_s = 16{\cdot}2$ by equation (ii); cf. 16·7 by equation (i)) were calculated. Of these, $\log K'$ may be considered reliable as it was given by seven consecutive portions of the titration; the other figures should be regarded as close approximations.

In an endeavour to measure the avidity of cobalt in the cobaltic-cysteine complexes, cysteine was titrated with cobaltous nitrate in a 3:1 ratio, no special precautions being taken to exclude oxygen. The solution remained red throughout and yielded two constants between pH 5 and 7 ($\bar{n} = 0{\cdot}4$–$1{\cdot}5$), namely, $\log K' = 9{\cdot}3$, $\log K'' = 7{\cdot}6$. Consistent results were not obtained at higher values of $\bar{n}$, hence $\log K'''$ could not be found. If, as an approximation, $\log K_s$ for a trivalent metallic ion is assumed to be 3 $\log K''$, then $\log K_s$ would be 23, the highest figure obtained for any amino-acid with any metal so far investigated.

Basic amino-acids

A homologous series of basic amino-acids is formed by diaminopropionic acid, diaminobutyric acid, ornithine and lysine (see Tables 2 and 3). A tendency is seen for type (II) rings to predominate among the lower isomers and type (I) rings among the higher isomers. This tendency is illustrated more clearly by Table 2 than can be done by merely comparing constants, because both types form, but to different extents. Comparison of $\log K_s$ values for glycine and ethylenediamine (Table 3) shows the superior complex-forming powers of two amino groups over an amino- and a carboxy-group (both substances give 5-membered rings). In all these diamino-acids the formation of type (I) complexes involves only 5-membered rings, whereas the type (II) rings become steadily larger (7-membered with ornithine, 8-membered with lysine). It is well known, from the examination of many types of chelate complexes, that high stability is found only in 5- and 6-membered rings. Hence the change from type (II) to type (I) at ornithine is readily understood.

αβ-Diaminopropionic and αγ-diaminobutyric acids. These gave type (II) complexes with Ni^{++}, Zn^{++} and Co^{++} throughout the titration range, 5- and 6-membered rings being formed respectively. In the case of diaminopropionic acid, copper appeared to begin by forming a type (I) complex of $\log K' = 6{\cdot}4$, up to a 1:1 ratio of amino-acid to metal. Thereafter a type (II) complex of $\log K' = 10{\cdot}8$ (approx.) and $\log K'' = 8{\cdot}0$ appeared (whence $\log K_s = 18{\cdot}8$ approx.). Another example of irregular combination with copper is discussed under histidine. Because of poor solubility the Fe^{++} titrations could not be followed far enough with any of the basic amino-acids (other than histidine) to allow of any conclusion being drawn as to their type. Inspection of Table 2 shows that copper is bound by αγ-diaminobutyric acid in a way that is intermediate between those of diaminopropionic acid and ornithine (which forms mainly a type (I) complex). A $\log K'$ of $7{\cdot}2 \pm 0{\cdot}07$ can be calculated for the type (I) part of its curve (i.e. up to the 1:1 complex). The remainder of the figures have not proved amenable to analysis: combinations according to types (I) and (II) are evidently proceeding simultaneously, the latter predominating.

Ornithine. This also gives both types of complexes, as can be gleaned from Table 2, but type (I) predominates. Copper, cobalt and zinc give steady constants when calculated according to type (I), but obviously a little type (II) formation is proceeding simultaneously. Nickel seems to combine largely according to type (I) with a $\log K_s$ of 8·3, but there is a substantial amount of type (II) combination at higher pH values (pH 7·5–10·5) with a $\log K_s$ of 10·3.

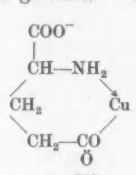
Lysine and arginine give ordinary type (I) complexes with hardly any recognizable amount of type (II) combination.

That the constants for diaminopropionic and

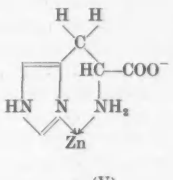
diaminobutyric acids tend to be stronger than those of ethylenediamine is to be expected from the coulombic attraction of the —COO^- for the metallic cations, so that these acids would be surrounded by a denser concentration of cations than could be present in the bulk of the solution. Similarly, the constants for ornithine, lysine and arginine are lower because the ω-amino groups, which are positively charged throughout the titration, repel the metallic cations. These ω-amino groups are likely to be brought close enough to the chelation site for this effect to operate by a bending of the molecule caused by coulombic attraction of these groups for the charged —COO^-. On the other hand, no such bending is to be expected in glutamic acid, which has the same number of carbon atoms as ornithine. This is because the ionic groups in the chelating species are all negatively charged; hence there is no increased affinity as compared with glycine. However, such an increase can be seen in aspartic acid in which the charges are closer.

Histidine, glutamic acid and aspartic acid

These three compounds form a special series where the pK_a of the 'other' group is intermediate between those of the two alpha groups (see Table 3). This introduces a new difficulty in the interpretation of the titration results when the end point coincides with the addition of 2 equivalents of alkali (as happens with these three substances). In such cases it is not immediately clear whether the 'other' group is involved in the chelation because it must be completely titrated in order to deionize any appreciable amount of the α-amino group which is essential for the chelation. Regardless of whether a type (I) or type (II) (type (IV) for glutamic acid) combination is occurring, equation (vii) must be used in its calculation because of the number of hydrogen ions liberated. Thus the choice of type has to be made on other grounds.



(IV)



(V)

Histidine. The choice between types (I) and (II) is made in favour of (II) from the magnitude of the values for Ni^{++}, Zn^{++} and Fe^{++} in Table 3, which are so much larger than the corresponding values for glycine (a type (I) combination should give values lower than those for glycine because of the coulombic repulsion of the glyoxaline cation). The actual form which type (II) takes for histidine is shown in (V). The log K_s values are higher than those of histamine (Table 3), just as those of diaminobutyric acid are higher than those of ethylenediamine (see above).

The combination with copper shows an irregularity that is quite the reverse of that exhibited by diaminobutyric acid. Here the titration is type (II) from its beginning and yields $\log K' = 10{\cdot}5$. On continuing the titration past $\bar{n} = 1{\cdot}0$, an (apparent) $\log K''$ of 8·3 is at first encountered. This gives $\log K_s$ of 18·8 from equation (ii), which agrees reasonably well with the value of 18·96 calculated by equation (i) from the mid-point. However, on continuing the titration past $\bar{n} = 1{\cdot}4$, $\log K''$ steadily declines to 6·5. The cause of this trouble is, partly, the cupric ion which, alone of the cations used in this work, is confined to a planar configuration in solution.

That copper can form non-symmetrical complexes must be borne in mind; for example, diethylenetriamine gives a K' corresponding to the union of a copper ion with the three nitrogen atoms of one molecule of amine, whereas the K'' corresponds to union with only one more nitrogen atom in a second molecule of the amine (Jonassen, LeBlanc & Rogan, 1950). On the other hand, this amine binds nickel symmetrically.

The combination of histamine and copper proceeds regularly throughout the titration so that the carboxyl group of histidine must be involved in the irregularity, in some way not yet known. The explanation must be accommodated within the framework of the loss of four hydrogen ions from two molecules of histidine because a sharp end point was obtained corresponding to 2 equivalents of alkali (as with nickel).

The histidine-cobaltous complex is known to absorb oxygen with great avidity, passing to the hexacovalent state, but remaining cobaltous (Hearon *et al.* 1949). Hence particular care was taken to exclude oxygen in this titration. The following results were obtained: $\log K' = 7{\cdot}1$, $\log K'' = 5{\cdot}6$, $\log K_s = 12{\cdot}7$ (from (ii)) and 12·6 (from (i)) with a good end point at 2 equivalents of alkali. Hearon *et al.* give 7·30, 4·42 and 11·62 for logs K', K'' and K_s respectively, but they describe the complexes as pink. In the present work, the solution was pink while the first equivalent of alkali was being added, and yellowed during addition of the second equivalent. The yellow solution could be back-titrated to the pink one and even if back-titrated to the starting point, the same pH figures were obtained. If the pink solution was shaken with air below pH 5·5 it did not change colour. (Hearon *et al.* describe the hexacovalent complex as 'deep amber'.) Such colour changes are most uncharacteristic of titrations involving cobalt and amino-acids. Thus a little uncertainty exists as to whether the complex remained tetracovalent throughout the

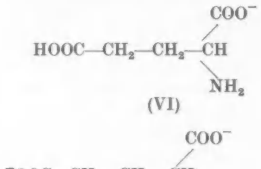
present work, and hence the results have been recorded here rather than in Table 3.

It is surprising that histidine, which binds most of these divalent metals with an avidity surpassed only by cysteine (among the amino-acids found in proteins), has less avidity than glycine for Mn^{++} and Mg^{++}.

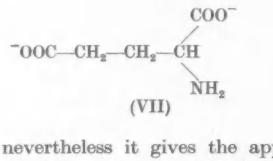
Maley & Mellor (1950) give the following log K_s values for the complexes of histidine with Cu^{++}, Co^{++}, Zn^{++} and Mn^{++} respectively: 18·3, 13·9, 12·9 and 7·7. The method of calculation is not given and no mention is made of the irregularities shown by Cu^{++} and Co^{++} during titration.

Glutamic acid. The end point coincides with the addition of 2 equivalents of alkali. For reasons given above, this fact alone gives no clue as to whether the combination is between the two α groups (type (I)), or between the γ-carboxyl group and the amino group (type (IV)) or even between the two carboxyl groups. These alternatives involve respectively 5-, 7- and 8-membered rings. It is evident from figures cited by Bjerrum (1950) that, in rings of equal size, two carboxyl groups do not bind metals so avidly as a carboxyl- and an amino-group. Thus the choice is between the 5- and the 7-membered ring and, as only 5- and 6-membered metal-containing rings have a high stability, it is concluded that glutamic acid forms a type (I) combination. The constants were calculated from equation (vii), for reasons given above.

Only in the case of copper is an irregularity encountered. This takes the form of an apparent log K' which falls from 9·7 ($\bar{n}=0{\cdot}2$) at pH 3·1 (where only 6 % of the glutamic acid is present as (VII)) to 8·4 ($\bar{n}=0{\cdot}8$) at pH 4·6 (where 67 % of the acid is present as (VII)). Although reliable calculations cannot be carried out above $\bar{n}=0{\cdot}8$, and the value may fall



(VI)



(VII)

further, nevertheless it gives the appearance of becoming steady and, when added to the log K'' figure for $\bar{n}=1{\cdot}2$, gives a log K_s of 14·7, in good agreement with the log K_s of 14·5 derived from equation (i). No trouble is encountered with log K'' ($=6{\cdot}3$) because practically no (VI) is present at the pH values where it is determined. Hence it would appear that 14·5 is a likely value for log K_s for the glutamic acid-copper complex.

Thus the initial irregularity in the combination of copper and glutamic acid is attributable to ionization of the γ-carboxyl group during the early part of the titration, (VI) becoming (VII). The lower affinity of the other metals permits this group to ionize almost completely before complex-formation occurs at the appropriately higher pH value. Aspartic acid is the only other amino-acid with a structure capable of causing this type of difficulty.

Aspartic acid. It is to be assumed that type (I) complexes are formed because the formation curves are parallel to those of glutamic acid. Aspartic acid gives higher constants than glycine, the anion of the γ-carboxy group being near enough to the chelating groups to attract the metallic cations more powerfully. Conversely, asparagine (where the γ-carboxamido group is electron attracting) gives lower constants than glycine (Part 1).

Because aspartic acid has a higher avidity than glutamic acid, the initial parts of both copper and nickel titrations show irregularities. Thus copper gives values of log K' descending from 10·2 ($\bar{n}=0{\cdot}3$) at pH 2·8 (7 % di-anion) to 9·1 ($\bar{n}=0{\cdot}8$) at pH 4·0 (56 % di-anion). Log K'' (6·8) is steady, as the ionization of the di-anion is complete throughout this part of the titration. Log K_s is approximately 15·7 by both tests given under glutamic acid (15·2 was found polarographically by Li & Doody, 1950). The irregularities in the nickel curve are similar but cease much earlier in the titration, 90 % di-anion being formed at $\bar{n}=0{\cdot}5$. Hence no difficulty arose in calculating the log K_s given in Table 3.

Greenwald (1939) found log $K'=2{\cdot}30$ for aspartic acid and magnesium.

Benzoylglutamic acid. This is of interest because it forms part of the folic acid molecule. Complex-formation should not involve both carboxyl groups, forming a 9-membered ring, but rather the α-carboxyl group and the nitrogen of the peptide linkage. With Cu^{++} (the only metal used), complex-formation was quite small. At pH 5·6, basic cupric chloride began to precipitate and at pH 7 complex-formation could no longer be demonstrated.

Ferric iron. In Part 1 no evidence could be found of an amino-acid binding ferric iron appreciably. The tendency of basic ferric salts to precipitate at about pH 3, unless a highly avid complex-forming substance is present, makes it difficult to detect lower, or even moderate, degrees of affinity.

It has been recorded that histidine, more than any other amino-acid, is capable of moving ferric iron along a paper chromatogram running in phenol (Gabrio & Tishkoff, 1950). However, the same pH readings were obtained on titrating ferric ammonium sulphate (0·0033 M), whether histidine hydrochloride (0·01 M) was present or not. Tyrosine and arginine behaved similarly. Aspartic acid gave a depression of 0·2 pH unit up to the point where precipitation

occurred (pH 2·7). Small as this depression is, it corresponds to $\log K' = 11{\cdot}0$ at this part of the titration. Glutamic acid gave a depression of only 0·07 pH unit. It is becoming clear that the avidity of substances for ferric salts can be measured only if there is no interfering pK_a between 1·5 and 3, or if the avidity is so great that basic ferric salts are not precipitated at pH 3.

Concluding remarks. In spite of the highly individual mode of union with metallic ions shown by many of the amino-acids examined here, no departure has been found from Mellor & Maley's order of avidity, discussed in Part 1. A different order, however, exists among the pteridines, the purines and riboflavine as will be shown in Part 3.

The amino-acids showed no departure from simple stepwise addition. The values for $\log K' - \log K''$ tend to lie between 1 and 2 (type (II) complexes tend to have higher values than types (I) or (III); the values for copper and nickel tend to be higher than those of the other metals, but are not proportional to $\log K_s$).

SUMMARY

1. The avidities of α-amino-acids with three ionizing groups for the ions of heavy metals have been measured and recorded as stability constants. For any one metal the stability constants varied over an enormous range (e.g. by 10^{11} for nickel). However, the order of avidities, for any given amino-acid, remains as with glycine (see Table 3).

2. Tyrosine, ornithine, lysine, arginine, aspartic acid and glutamic acid were found to combine through the α-amino- and α-carboxyl-groups, after the manner of glycine. Other acids combine through the α- and the ω-nitrogen atoms (or, in the case of cysteine, the sulphur atom). The homologous series: lysine, ornithine, αγ-diaminobutyric acid and αβ-diaminopropionic acid has been examined and found to yield examples of transition between these two extremes (see Tables 2 and 3).

3. Several of the acids show abnormalities of behaviour with copper; cysteine and histidine show anomalies with cobalt.

4. The metal-binding properties of histamine have been studied for comparison with those of histidine.

5. The ionization constants of αγ-diaminobutyric, αβ-diaminopropionic and benzoylglutamic acids have been determined for the first time.

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APPENDIX

Derivation of Equations

By J. N. PHILLIPS

A. *Derivation of equation for* $\bar{n}$

In the simple case where only two ionizing groups are present, the total concentration of complexing agent $[XSc^\circ]$ is the sum of five concentrations:

$$[XSc^\circ] = [Sc] + [ScH^+] + [ScH_2^{++}] + [ScM] + 2\,[Sc_2M]. \quad \text{(viii)}$$

But, by definition, $\bar{n}$ is the average number of molecules of complexing agent bound per atom of the metal, i.e.

$$\bar{n} = \frac{[ScM] + 2\,[Sc_2M]}{[M^\circ]}. \quad \text{(ix)}$$

Hence

$$\bar{n}\,[M^\circ] = [ScM] + 2\,[Sc_2M]. \quad \text{(x)}$$

Substituting (x) into (viii) gives

$$[XSc^{\circ}]=[Sc]+[ScH^{+}]+[ScH_2^{++}]+\bar{n}\,[M^{\circ}]. \quad \text{(xi)}$$

Now, in the Mass Action equations (xii),

$$K_a=\frac{[H^+]\,[A^-]}{[HA]}, \quad \text{and} \quad K'_a=\frac{[H^+]\,[HA]}{[H_2A^+]}, \quad \text{(xii)}$$

which describe the ionization of a dibasic acid, $[A^-]$ corresponds to [Sc], [HA] to $[ScH^+]$, etc. Hence

$$[ScH_2^{++}]=\frac{[H^+]^2\,[Sc]}{K_aK'_a} \quad \text{and} \quad [ScH^+]=\frac{[H^+]\,[Sc]}{K_a}. \quad \text{(xiii)}$$

Substituting (xiii) into (xi) gives

$$[XSc^{\circ}]=[Sc]+\frac{[H^+]^2\,[Sc]}{K_aK'_a}+\frac{[H^+]\,[Sc]}{K_a}+\bar{n}\,[M^{\circ}], \quad \text{(xiv)}$$

rearrangement of which gives

$$\bar{n}=\frac{[XSc^{\circ}]-[Sc]\left\{\frac{[H^+]^2}{K_aK'_a}+\frac{[H^+]}{K_a}+1\right\}}{[M^{\circ}]} \quad \text{(xv)}$$

$$=\frac{[XSc^{\circ}]-\alpha\,[Sc]}{[M^{\circ}]}. \quad \text{(xvi)}$$

Where three ionizing groups are present, the term

$$\frac{[H^+]^3}{KK'_aK''_a}$$

is added to α.

B. *Derivation of equation for* [Sc]

The concentration of free and combined hydrogen ions $[XH^+]$ can be expressed in two ways, (xvii) and (xviii):

$$[XH^+]=[H^+]+[ScH^+]+2\,[ScH_2^{++}]-[OH^-], \quad \text{(xvii)}$$

$$[XH^+]=[XSc^{\circ}]+[HCl]-[KOH], \quad \text{(xviii)}$$

where [HCl] and [KOH] equal the concentrations which these reagents would reach if run into the same volume of pure water. Combining (xvii) and (xviii),

$$2\,[ScH_2^{++}]+[ScH^+]=[XSc^{\circ}]+[HCl]-[KOH]-[H^+]+[OH^-]. \quad \text{(xix)}$$

Substituting terms from equation (xiii), and rearranging,

$$[Sc]=\frac{[XSc^{\circ}]+[HCl]-[KOH]-[H^+]+[OH^-]}{\frac{2\,[H^+]^2}{K_aK'_a}+\frac{[H^+]}{K_a}} \quad \text{(xx)}$$

$$=\frac{[XSc^{\circ}]+[HCl]-[KOH]-[H^+]+[OH^-]}{\beta}. \quad \text{(xxi)}$$

[HCl] and [KOH] are alternative terms, depending on which reagent was used in the titration. In general $[OH^-]$ becomes significant only above pH 10. Where three ionizing groups are present, the term $\frac{3\,[H^+]^3}{K_aK'_aK''_a}$ is added to β.

Photometric Micro-analytical Estimation of Acetic Acid in Blood and Tissues

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The chemical methods which have been proposed for the estimation of acetic acid in biological materials are not specific and are, in consequence, of restricted value. Treatment of acetic acid with cupric oxide yields oxalic acid, and this reaction was utilized by Mugdan & Wimmer (1933) for the estimation of acetic acid; but, under their conditions tartaric, glycollic and the lower fatty acids also yield oxalic acid. Pyrolysis of calcium acetate yields acetone, but since the calcium salts of other monocarboxylic acids also yield carbonyl compounds under these conditions, the method of Dulian (1939), which is based on this reaction, is inadequate. A photometric method based on the lanthanum reaction of Kruger & Tschirch (1929) has recently been developed by Hutchens & Kass (1949), but since propionic acid also gives the blue colour their method lacks specificity.

Procedures dependent on the physical properties of acetic acid can be classified into: (i) Distillation methods: Duclaux (1900), Dyer (1917), Olmsted, Whitaker & Duden (1929), McClendon (1944) and Black (1949). (ii) Partition methods: Behrens (1926), Werkman (1930*a*, *b*, *c*, 1931). Friedemann (1938) devised a method which is a combination of both the distillation and partition methods. (iii) Chromatographic methods: Ramsey & Patterson (1945, 1948), Elsden (1946), Ramsey (1948), Moyle, Baldwin & Scarisbrick (1948) and Peterson & Johnson (1948). Of all these procedures only those involving chromatography can make any real claim either to specificity or to precision.

Acetic acid, however, does differ from the other members of the homologous series in its stability to the potassium dichromate-sulphuric acid reagent; but at the same time it is completely oxidized by treating it with a silver dichromate-sulphuric acid mixture (Simon, 1922*a*, *b*). Cordebard & Michl (1928) used this difference in behaviour as the basis of a quantitative method for the estimation of acetic acid in known mixtures; their method was adapted to the micro-estimation of acetic acid by Caselli & Ciaranfi (1942). In this procedure acetic acid was separated from its higher homologues by conversion

to the methyl ester and subsequent distillation. The interfering compounds which distilled with methyl acetate were allowed for by treating with potassium dichromate-sulphuric acid prior to oxidation with the silver dichromate-sulphuric acid reagent. This method, whilst useful for known mixtures, was not directly applicable to the estimation of acetic acid in biological materials. It has, however, proved possible to modify the technique in such a way as to permit the photometric estimation of acetic acid in blood and tissues.

METHOD

Principle. Methyl acetate (b.p. 57°) boils at a lower temperature than methanol (b.p. 65°), whereas the methyl esters of the other members of the homologous series have a higher boiling point; consequently by esterifying a mixture of fatty acids in excess methanol and subsequent distillation it is possible to effect a considerable separation of acetic acid from the remainder. After one such distillation and with the relative recovery of acetic acid placed at 100, the recoveries of other acids are as follows: formic, 87; propionic, 63; butyric, 44; valeric, 8; hexanoic, 3; heptanoic, 1. The recovery of formic acid is not quantitative as it is partially decomposed during esterification in the presence of H_2SO_4. H_2S, produced from cysteine and related compounds, is converted to methyl mercaptan and distils under these conditions. By repeated distillation methyl mercaptan, methyl formate and methyl acetate are separated from the methyl esters of the higher acids, and saponification of the esters in the final distillate yields the sodium salts of H_2S, formic and acetic acids. The first two are readily oxidized by treatment with the $K_2Cr_2O_7$-H_2SO_4 reagent, whereas acetate is oxidized only in the presence of Ag^+.

Under standard conditions the extra dichromate used following treatment with $Ag_2Cr_2O_7$ is related to the amount of acetate present. The dichromate may be estimated either photometrically, using a filter absorbing at 450 mμ., or titrimetrically.

Reagents. Methanol (A.R.). Traces of volatile acids are removed by adding 2 g. of NaOH/l. of methanol and distilling from an all-glass apparatus. Conc. H_2SO_4 (sp.gr. = 1·84). NaOH (40%, w/v, and 1·25%, w/v). Saturated aqueous solution of $AgNO_3$. 0·05 N-$K_2Cr_2O_7$.

Apparatus. (*a*) Distillation units, seven of which are required. These are constructed of 8 mm. bore tubing and are of the micro-Kjeldahl type as modified by Embden. The ascending limb is 15 cm. long with a bulb of 3 cm. diameter situated 5 cm. above the standard joint. The oblique limb is 20 cm. long and the descending limb 40 cm.; the first 18 cm. of the latter is water-jacketed.

(*b*) A supply of 50 and 100 ml. Kjeldahl flasks fitted with standard joints. The 50 ml. flasks carry a 12 ml. calibration mark.

(*c*) Two Schiff-type reflux condensers 80 cm. long and fitted with standard joints.

(*d*) A Coleman spectrophotometer model S-14 with round cuvettes (19 × 105 mm.) or a Lumetron photoelectric colorimeter model 400-A.

Extraction. Blood. Blood (5 ml.) is placed in a centrifuge tube containing 40 ml. of cold methanol, stirred thoroughly with a glass rod and centrifuged at 5000 rev./min. for 5 min. in a refrigerated centrifuge. The supernatant is transferred to a measuring cylinder and the volume noted. It is filtered into a 100 ml. Kjeldahl flask and both cylinder and filter paper are washed with a few ml. of methanol, the washings being added to the Kjeldahl flask.

Tissues. A known weight of tissue (approx. 1 g. when using liver, kidney or muscle) is placed in a cooled glass mortar and ground thoroughly with powdered quartz sand; 5 vol. of cooled methanol are then added and stirred with a glass rod. The mixture is transferred to a centrifuge tube and the mortar washed with a further 5 vol. of cooled methanol and the washings added to the centrifuge tube. The contents are made alkaline with 0·2 ml. of 40% (w/v) NaOH and procedure continued as for blood.

Analytical procedure. The Kjeldahl flask containing the methanolic extract is attached to a distillation unit and the contents boiled gently in order to minimize frothing. When the volume has been reduced to about 3 ml. the flask is disconnected and the contents taken to dryness in a bath of boiling saturated $CaCl_2$, the vapours being removed with a water pump. The flask is then cooled and 15·0 ml. methanol and 1·0 ml. conc. H_2SO_4 added. The ground-glass joint is lubricated with a few drops of conc. H_2SO_4 and the flask attached to a second distillation unit. Methanol (4·0 ml.) is added to a 50 ml. Kjeldahl flask, which serves as a receiver, and which is so placed that the tip of the condenser dips below the surface of the methanol. It is distilled gently until the volume in the receiver is 12 ml.; the tip of the condenser is rinsed with 4·0 ml. methanol which are added to the rinsings in the receiver. During this step the fatty acids present in the extract are esterified and the esters distilled over. The methyl acetate is next separated from the esters of the higher acids by repeated distillation; in all, five such distillations are required for complete separation (Table 1). In each case distillation is stopped when the volume in the 50 ml. Kjeldahl flask is 12 ml. and the tip of the condenser washed down with 4 ml. methanol. The final distillate is

Table 1. *Elimination of C_3–C_7 fatty acids by successive distillations of their methyl esters*

Acid	Amount added (mg.)	Amount* (mg.) recovered after					
No. of distillations ...		1	2	3	4	5	6
Formic	1·00	0·88	0·86	0·90	0·84	0·85	0·90
Acetic	1·00	0·98	1·05	1·02	1·00	1·01	1·03
Propionic	1·00	0·63	0·41	0·29	0·15	0·04	— †
Butyric	1·00	0·44	0·22	0·11	—	—	—
Valeric	1·00	0·08	—	—	—	—	—
Hexanoic	1·00	—	—	—	—	—	—
Heptanoic	1·00	—	—	—	—	—	—

* Mean values of several determinations. Individual determinations ranged within ±5% of the mean.
† — signifies that the fatty acid amount was not measurable.

collected in 4·0 ml. of 1·25% (w/v) NaOH instead of methanol. A glass bead is added and the mixture set aside for 30 min. to ensure complete hydrolysis of the esters. The contents of the flask are then taken to dryness in the boiling $CaCl_2$ bath. The dry sodium salts are next treated with the oxidizing agents as follows. 0·05 N-$K_2Cr_2O_7$ (7·0 ml.) is added followed by 7·0 ml. conc. H_2SO_4, the latter being added in such a way as to moisten the standard joint. The flask is attached to a reflux condenser and the contents boiled for 30 min. When the flask has cooled 4·0 ml. of the contents are removed with a pipette and delivered into a colorimeter tube containing 6·0 ml. water; this sample is called 'K unknown'. Two drops of saturated $AgNO_3$ and 3·0 ml. conc. H_2SO_4 are added to the remaining liquid in the flask and the contents boiled under a reflux condenser for

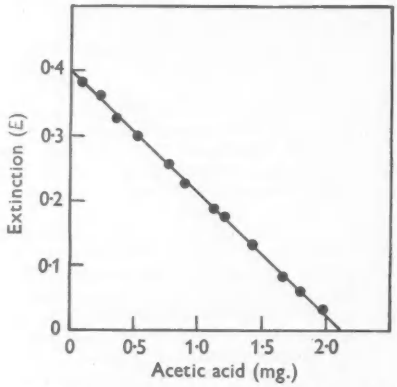

Fig. 1. Calibration curve obtained by estimating samples of standard acetic acid solution, dried together with 4·0 ml. of 1·25% (w/v) NaOH.

1 hr. When cool, a second 4·0 ml. sample is transferred to a colorimeter tube containing 6·0 ml. water; this sample is called 'Ag unknown'. Whilst carrying out the oxidation of the unknown a reagent blank is performed and the two 4·0 ml. samples taken are referred to as 'K blank' and 'Ag blank' respectively. The amount of dichromate present in each sample is then determined spectrophotometrically. If a suitable spectrophotometer is used such as one of those specified it is possible to correct for the blanks directly on the instrument. In all there are three corrections to be made, the 'K blank' and the 'Ag blank' and, in addition, that due to materials present in the distillate which are oxidized by dichromate in the absence of Ag^+, i.e. formate and sulphide.

If the amount of dichromate taken has been sufficient to oxidize all the acetate present then, by setting the 'Ag blank' at an extinction of 0·4 the 'Ag unknown' will give a reading of between 0·4 and 0·0. If this is the case, the 'K unknown', which corresponds to zero acetate concentration, is set at an extinction of 0·4 and the extinction of the 'K blank' determined. This is equal to or greater than 0·4 depending on the amount of easily oxidizable material (formate and sulphide) present in the sample. The reading of the 'K blank' so obtained is used as the arbitrary zero against which the extinction of the 'Ag unknown' is measured. Fig. 1 gives the calibration curve of the method showing that there is a linear relationship between acetic acid and extinction over the range 0·0–2·3 mg. acetic acid.

Titrimetric method. If a photometer is not available a titrimetric procedure similar to that of Caselli & Ciaranfi (1942) may be used. In addition to the apparatus and reagents specified for the photometric procedure the following solutions are required:

0·05 N-$FeSO_4(NH_4)_2SO_4.6H_2O$ in 5% (v/v) H_2SO_4,

0·05 N-$KMnO_4$.

The extraction and distillation procedures are the same as those outlined above. However, the final distillate, which is collected in 4·0 ml. 1·25% (w/v) NaOH solution, is treated differently. The distillate is divided into two equal parts each of which is transferred to a 50 ml. Kjeldahl flask. After allowing 30 min. to complete the hydrolysis of the esters the contents of each flask are taken to dryness. To one flask is added 5·0 ml. 0·05 N-$K_2Cr_2O_7$ and 5·0 ml. conc. H_2SO_4 and to the other 5·0 ml. 0·05 N-$K_2Cr_2O_7$, two drops saturated $AgNO_3$ and 8·0 ml. conc. H_2SO_4. Each flask is then boiled for 1 hr. under a reflux condenser and, when cool, 5·0 ml. of 0·05 N-ferrous ammonium sulphate added, and the contents titrated with 0·05 N-$KMnO_4$. In addition, both a 'K blank' and an 'Ag blank' are determined. When acetate is oxidized under these conditions, 1 ml. of 0·05 N-dichromate is equivalent to 0·375 mg. acetic acid. The amount of acetic acid present in half the sample of blood or tissue taken is equal to $[(A-a)-(K-k)]$ 0·375 mg., where A, a, K and k are the volumes of 0·05 N-$KMnO_4$ required for the 'Ag unknown', 'Ag blank', 'K unknown' and 'K blank' respectively.

RESULTS

Recoveries of known amounts of acetic acid added to various tissues as determined by the photometric and the titrimetric procedures are given in Tables 2 and 3 respectively; Table 4 gives the results of estimations of the acetic acid content of blood, liver,

Table 2. *Recovery of acetic acid estimated photometrically*

('Found' values are corrected for acetic acid present in controls.)

Material	Acetic acid Added (mg.)	Acetic acid Found (mg.)
Blood (human)	0·13	0·13
	0·26	0·24
	0·39	0·36
	0·78	0·84
	1·17	1·15
	1·30	1·24
	1·43	1·36
	1·95	1·90
Muscle (rat)	0·37	0·40
	0·74	0·72
Kidney (rat)	0·70	0·72
	1·11	1·08
Kidney (guinea pig)	0·30	0·32
	0·39	0·38
	0·56	0·53
	1·26	1·21
Liver (guinea pig)	0·39	0·39
	0·61	0·58

Table 3. *Recovery of acetic acid estimated volumetrically from human blood*

('Found' values are corrected for acetic acid present in controls.)

Acetic acid	
Added (mg.)	Found (mg.)
0·13	0·14
0·22	0·23
0·25	0·25
0·50	0·52
0·51	0·48
0·53	0·51
0·54	0·58
0·56	0·55
0·63	0·61
1·00	0·94

Table 4. *Acetic acid determinations in blood and tissues*

		Acetic acid found (mg./100 ml. for blood, mg./100 g. for tissues)		
	Material	Mean	Range	No. of determinations
Blood	Human venous*	4·8	3·2–7·2	10
	Sheep arterial*	7·1	4·6–8·8	3
	Guinea pig arterial†	10	8·2–12·0	3
	Rat arterial†	9	6·1–12·0	3
Liver	Guinea pig†	61	35–98	5
	Rat†	54	40–69	3
Kidney	Guinea pig†	47	27–71	3
	Rat†	35	20–52	3
Muscle	Guinea pig†	23	11–36	3
	Rat†	16	10–22	3

* 6 hr. after feeding. † 1 hr. after feeding.

kidney and muscle. Reid (1950) determined the acetic acid content of sheep blood using the chromatographic methods of Elsden (1946) and of Moyle *et al.* (1948), and our figures for sheep blood are in agreement with those obtained by this author. On the other hand, McClendon (1944), in his paper on the micro-estimation of total volatile fatty acids in biological materials, states that the venous blood of both normal humans and dogs is 0·0003 N in respect of these acids; our results for acetic acid alone are substantially higher. Unfortunately, data are lacking for the acetic acid content of other tissues using other methods, and we are thus unable to obtain a comparison of our method.

SUMMARY

1. Acetate, extracted from blood or tissues with methanol, is dried and transformed into methyl acetate. By repeated distillations the C_3–C_7 fatty acid esters are removed. The ester is saponified and the resulting sodium acetate brought to dryness. Formic acid and hydrogen sulphide, which cannot be eliminated in such a way, are oxidized by potassium dichromate-sulphuric acid, the acetic acid remaining unchanged. Then acetic acid is fully oxidized by increasing the sulphuric acid concentration and by adding silver ions. The reduction of dichromate is measured photometrically and the acetic acid is estimated by the difference in the extinction.

2. This method permits the estimation of acetic acid in blood and tissues (0·1–2·3 mg.) with sufficient accuracy for biological purposes. The error is always less than ± 10 %.

3. The concentrations of acetic acid under physiological conditions, in human blood and in the blood, liver, kidney and muscle of the guinea pig and rat are discussed.

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Studies in Detoxication

42. FLUOROBENZENE. SPECTROPHOTOMETRIC DETERMINATION OF THE ELIMINATION OF UNCHANGED HALOGENOBENZENES BY RABBITS. A COMPARISON OF THE OXIDATION *IN VIVO* OF FLUOROBENZENE AND OF BENZENE

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Fluorobenzene is very similar in odour and general properties to benzene and, in fact, it resembles benzene more closely than it does the other halogenobenzenes. We shall show in the present paper that this similarity is also evident during its metabolism, for the main metabolites of fluorobenzene are excreted in amounts which are quantitatively similar to those of benzene rather than the other halogenobenzenes.

The earlier work on fluorobenzene centred on its effect on sulphur metabolism, and Coombs (1927) showed that in the dog, fluorobenzene caused a rise in the neutral sulphur output of the urine. This rise, however, was much smaller than that observed with the other monohalogenobenzenes and Coombs suggested that this could be in part due to the greater volatility of fluorobenzene. Coombs's work suggested that fluorobenzene formed a mercapturic acid *in vivo* and this was confirmed when Young & Zbarsky (1944) isolated *p*-fluorophenylmercapturic acid from the urine of rats which had received fluorobenzene orally or subcutaneously. The yields of mercapturic acid in Young & Zbarsky's experiments were about 1–2 % of the dose, which is small when compared with the amounts (25 % of the dose) which arise from chloro-, bromo- and iodo-benzenes (see for example, Spencer & Williams, 1950). Recently we have shown that, in the rabbit, benzene yields only 1–2 % of mercapturic acid (Parke & Williams, 1951) and the present work suggests the same order of mercapturic acid output in these animals when fluorobenzene is administered.

We shall also show that the halogenobenzenes can be estimated in a very simple manner by means of their light absorption in the ultraviolet.

EXPERIMENTAL

Spectrophotometric determination of halogenobenzenes

Previous methods for the estimation of halogenobenzenes include combustion and determination of the gaseous products (Thomas, Ivie, Abersold & Hendricks, 1943), the determination of the colour imparted to flames by $CuCl_2$ (Department of Scientific and Industrial Research, 1940) and nitration followed by colorimetric determination of dinitrohalogenobenzenes (Ginzberg, 1947). The spectroscopic method developed here appears to surpass all these methods in accuracy and simplicity.

Materials. The halogenobenzenes (British Drug Houses Ltd.) were redistilled before use and had the following boiling points (uncorr.): fluorobenzene, 83°; chlorobenzene, 130°; bromobenzene, 154°; and iodobenzene, 186°. Ethanol was of 'anhydrous for spectroscopy' grade. Spectrographic measurements were made with a Unicam Spectrophotometer, Model SP500.

Light absorption data. The absorption spectra of the four halogenobenzenes in ethanol are given in Fig. 1. The spectra of these compounds in hexane have been previously determined by Conrad-Bilroth (1932, 1934). Klingstedt (1933) has recorded the spectrum of iodobenzene in hexane, and Morton & Stubbs (1940) have quoted figures for chlorobenzene in ethanol.

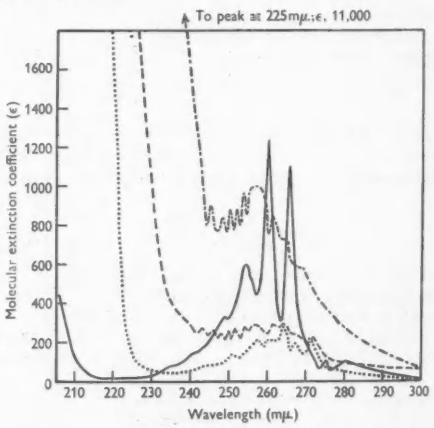

Fig. 1. Absorption spectra of fluorobenzene (—), chlorobenzene (. . . . .), bromobenzene (– – –) and iodobenzene (–·–·–) in ethanol (see also Table 1).

In the present work the selected maxima and their extinctions for the quantitative estimation of the halogenobenzenes are given in Table 1. Since the spectra of these compounds show a number of sharp peaks close together the slit width of the spectrophotometer at which the extinctions

of the maxima are determined is of great practical importance. When the molecular extinction coefficient has been determined for the pure compounds at a given slit width, the same slit width must then be used for the determination of all unknown solutions. Extinction values for the halogenobenzenes in the 260 mμ. region were found to vary appreciably with the slit width of the instrument. Data for two values of the slit width are given in Table 1.

Table 1. *Wavelengths and extinctions of absorption bands selected for the determination of halogenobenzenes in ethanol*

	Slit width 0·2 mm.		Slit width 0·5 mm.	
Compound	$\lambda_{max.}$ (mμ.)	$\epsilon_{max.}$	$\lambda_{max.}$ (mμ.)	$\epsilon_{max.}$
Fluorobenzene	260·25	1,250	260	700
Chlorobenzene	264	280	263·5	170
Bromobenzene	264	300	263·5	197
Iodobenzene	225	11,000*	224·5	10,000†
	256·7	1,000	257	660

* Slit width 1·0 mm. † Slit width 1·5 mm.

The intense bands at 210–220 mμ. which occur in the spectra of fluoro-, chloro- and bromo-benzene were not suitable for accurate estimation of these compounds, since the spectrophotometer is not sufficiently reliable in this region. In the case of iodobenzene these particular bands are shifted to longer wavelengths and the peak at 225 mμ. is at a point where the instrument is reliable. The selected wavelengths of all four compounds were shown to obey the Lambert-Beer Law within the recommended scale readings ($E = 0{\cdot}3$–$0{\cdot}8$) of the spectrophotometer.

Apparatus. The tank used for the analysis of the expired air of rabbits was identical with that described by Parke & Williams (1950) for the study of the elimination of unchanged benzene by rabbits. The absorption train consisted of four Dreschel bottles, the first two (nearest the tank) containing a layer of glass wool on top of which was placed anhydrous $CaCl_2$ (14–20 mesh) to absorb water vapour and thus prevent ice formation. The recovery experiments quoted in Table 2 show that no halogenobenzene was lost in these bottles. The next two bottles each contained 40 ml. of 2 mm. glass beads and 25 ml. of ethanol and were kept throughout an experiment at −50° in a freezing mixture of solid CO_2 and acetone. All connexions were made of 'Portex' polyvinyl plastic tubing (Portland Plastics Ltd., 6 Victoria Street, London, S.W. 1), and ground-glass joints were lubricated with silicone high vacuum grease (Dow Corning Corporation, Midland, Michigan, U.S.A.).

Air was drawn through the apparatus at the rate of 50 l./hr. and the ethanol absorption bottles were renewed about every 2 hr. The halogenobenzene in the absorption bottles was estimated by pouring the ethanol and glass beads into a sintered-glass filter, washing the beads with ethanol and collecting the filtrate. The filtrate was then made up to 200 ml. at 20° with ethanol. Suitably diluted portions were then examined in the spectrophotometer in 1 cm. quartz cells.

Recovery of halogenobenzenes. Weighed amounts of the halogenobenzenes were introduced and spilt in the tank (see Parke & Williams, 1950). The latter was sealed and a current of air drawn through it. The amount of halogenobenzene was then estimated as above (see Table 2). The laboratory atmosphere could be simultaneously tested by means of a Y-piece placed between the absorption train and the tank. We never found any significant amounts of hydrocarbons or interfering materials in the laboratory atmosphere. The amounts of the halogenobenzenes used in these recovery experiments were those expected in our animal experiments.

Animal experiments

The animals used were Chinchilla doe rabbits weighing about 3 kg. These animals were kept on a diet of 70 g. rat cubes and 150 ml. water/day. The dose of halogenobenzene was administered by stomach tube (made of polyvinyl plastic) as an emulsion in water. In some experiments with fluorobenzene, the undiluted compound was given by intraperitoneal injection. Immediately after administering the dose, the animal was placed in the tank which was sealed and connected to the absorption train. A current of air, at the rate of 50 l./hr., was then drawn through the whole apparatus until the amount of halogenobenzene eliminated was negligible; this usually took about 30 hr. or more. The absorption bottles were changed at frequent intervals and the halogenobenzene determined spectroscopically as above. A complete absorption spectrum was determined on several samples of the absorption fluid during the course of each experiment to prove that the material being determined was

Table 2. *Recoveries of halogenobenzenes from the tank estimated spectrophotometrically*

			Weight recovered			
Compound	Wt. introduced into tank (g.)	Duration of aeration (hr.)	1st bottle (g.)	2nd bottle (g.)	Total (g.)	Total (as % of wt. introduced)
Fluorobenzene	1·024*	5	1·020	0·002	1·022	100
	1·024*	6	1·015	0·008	1·023	100
	0·521	4	0·524	0·002	0·526	101
	0·516	5	0·500	0·000	0·500	97
Chlorobenzene	0·338	5	0·327	0·001	0·328	97
	0·298	4·5	0·293	—	0·293	98
Bromobenzene	0·0663	6·5	0·0655	—	0·0655	99
	0·0672	6·5	0·0658	0·002	0·0678	101
Iodobenzene	0·0411	5	0·0390	0·0001	0·0391	95
	0·0412	7	0·0403	0·0009	0·0412	100

* In these instances the fluorobenzene was recovered from a Dreschel bottle.

the halogenobenzene fed and on every occasion this was done; the spectrum obtained was that of the substance fed. Table 3 is a summary of the results and Fig. 2 shows graphically the elimination of fluorobenzene in a single experiment.

Table 3. *Elimination of unchanged halogenobenzenes in the expired air of rabbits receiving these compounds orally*

Compound fed	Rabbit no.	Dose (g./kg.)	Duration of experiment (hr.)	Unchanged compound eliminated (as % of dose)
Fluorobenzene	37	0·5	31	41
	37	0·5†	50	41
	66	0·5	30	32
	68	0·5	32	53
	72	0·5	27	49
	66	0·5	10*	31*
	67	0·5	12*	43*
	37	1·0	30	73
	66	1·0	42	68
	68	1·0	32	57
	72	1·0	48	59
Chlorobenzene	37	0·5	30	32
	68	0·5	30	24
	71	0·5	30	25
Bromobenzene	71	0·5	26	5·5
	72	0·5	26	7·0
Iodobenzene	37	0·5	30	3·8
	72	0·5	30	2·7

* Incomplete experiments.
† Injected intraperitoneally.

Experiments on the oxidation of fluorobenzene

Qualitative tests on the urine from rabbits fed with fluorobenzene showed the presence of fluorophenols, and the extent of their formation was assessed by determinations of ethereal sulphate and glucuronic acid in fluorobenzene urine.

Glucuronic acid was determined by the method of Hanson, Mills & Williams (1944) using pure D-glucurone as standard. The addition of 0·1 mg. of $K_2S_2O_8$ in 0·1 ml. water to each tube prior to immersion in the boiling-water bath was found to improve the method.

Ethereal sulphate was determined by the turbidimetric method of Sperber (1948) with the modification that the standard curve was made with $(NH_4)_2SO_4$, not in aqueous solution, but in diluted (1:4) rabbit urine. The standard curve in rabbit urine was not the same as in aqueous solution. Using this method, the recovery of potassium *p*-chlorophenylsulphate (0·5–1·5 mg. in 2 ml. urine) was 98–105%.

Mercapturic acid was determined iodometrically (Stekol, 1936; cf. Parke & Williams, 1951). We have not proved, however, that the urine of rabbits receiving fluorobenzene contains a mercapturic acid and therefore our figures for mercapturic acid are only given tentatively, although no other metabolite has been found which consumes iodine under the conditions of the estimation.

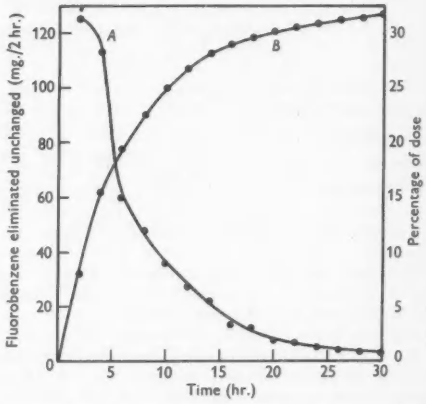

Fig. 2. The elimination of unchanged fluorobenzene in the expired air of rabbit no. 66 which had received 1·536 g. of fluorobenzene orally. *A*, curve showing the amount of fluorobenzene in mg. eliminated unchanged in each 2 hr. period after dosing. *B*, curve showing the total amount of fluorobenzene eliminated as a percentage of the dose.

The results of the above determinations are given in Table 4. This table shows that the total *O*-conjugation of fluorobenzene is about 30% of the dose. With rabbits nos. 32, 39 and 55 some observations were made on the effect of the time of giving normal food on the glucuronic acid and ethereal sulphate conjugations (cf. Bray & White, 1951). In Exps. 1, 3 and 5 the animals were allowed access to their food immediately after dosing with fluorobenzene, whereas

Table 4. *Effect of fluorobenzene on the glucuronic acid, ethereal sulphate and mercapturic acid output of rabbits*

Exp. no.	Rabbit no.	Dose (g./kg.)	Extra glucuronic acid, *G* (as % of dose)	Extra ethereal sulphate, *E* (as % of dose)	*E* + *G*	Apparent 'mercapturic acid'* (as % of dose)
1	55	0·8	6·7	25·5	32·2	1·5
2	55	0·8	12·6	22·9	35·5	1·9
3	39	0·8	6·2	21·1	27·3	1·5
4	39	0·8	10·8	17·8	28·6	1·8
5	32	0·7	4·8	26·8	31·6	1·4
6	32	0·7	10·9	24·1	35·0	1·6
7	51	0·5	12·0	14·8	26·8	—
8	53	0·5	13·7	18·7	32·4	—
9	68	0·5	13·2	18·5	31·7	—

* By iodometric titration (see above).

in Exps. 2, 4 and 6 the animals were given their food 4 hr. after dosing. In these experiments the time of giving food had no effect on the total conjugation, but in those cases where food was allowed immediately after dosing with fluorobenzene there was an increase in ethereal sulphate conjugation and a decrease in glucuronic acid conjugation. In Exps. 7, 8 and 9, the time of giving food was not recorded. Much, of course, depends in this type of experiment upon whether or not an animal has lost its appetite temporarily after dosing with foreign compounds. With fluorobenzene the animals did not lose their appetite as they did after dosing with benzene.

Experiments on the oxidation of benzene

The ethereal sulphate and glucuronic acid outputs of rabbits receiving benzene have been determined by Porteous & Williams (1949). In order to compare fluorobenzene with benzene under exactly the same conditions, the glucuronic acid and ethereal sulphate outputs of rabbits receiving benzene have again been determined. The methods used were those described in the preceding section. The results are given in Table 5.

Table 5. *Effect of an oral dose of benzene on the glucuronic acid and ethereal sulphate output of rabbits*

(Dose, 0·5 g./kg.)

Rabbit no.	Extra glucuronic acid, *G* (as % of dose)	Extra ethereal sulphate, *E* (as % of dose)	*G* + *E*
51	8·8	17·1	25·9
53	8·7	22·3	31·0
68	20·4	32·0	52·4
75	12·8	15·3	28·1
82	10·4	30·1	40·5
87	6·4	31·4	37·8

In these experiments the animals were allowed access to their food immediately after dosing with benzene, but in many cases the animals had temporarily lost their appetite.

DISCUSSION

Our results show that a considerable amount of fluorobenzene when fed by mouth is eliminated in the expired air in the unchanged state, for at dose levels of 0·5 and 1·0 g./kg., 44 and 64 % of the dose respectively are so eliminated. Fluorobenzene is thus similar to benzene in this respect (see Parke & Williams, 1950). The other halogenobenzenes are also eliminated to some extent in the expired air. Chlorobenzene, despite its much higher boiling point and much lower vapour pressure, is eliminated unchanged to the extent of 25–30 %. Even the high-boiling bromo- and iodo-benzenes are also eliminated to a small but appreciable extent in the expired air (see Tables 3 and 6). In fact, more of the chloro-, bromo- and iodo-benzenes is eliminated than would be expected from their high boiling points and low vapour pressures. This suggests the possibility that they are eliminated by a process analogous to steam distillation which depends on molecular weight as well as vapour pressure. It is to be noted that the elimination of the halogenobenzenes at a dose level of 0·5 g./kg. takes about 30–40 hr. In one instance fluorobenzene was injected and some 50 hr. was needed for the elimination of the unchanged material (Table 3).

Some 30 % of fluorobenzene is also oxidized *in vivo* and about one-third of the oxidized fluorobenzene appears as glucuronide and two-thirds as ethereal sulphates (Table 4). Preliminary experiments have shown that the oxidized forms are fluorophenols. The quantitative aspects of fluorobenzene oxidation are very similar to those of benzene (compare Tables 4 and 5 and see summary in Table 7). The scatter in the figures for benzene is larger than that found in those for fluorobenzene. Porteous & Williams (1949) also reported a considerable scatter in their figures for the glucuronic acid and ethereal sulphate output of rabbits receiving benzene.

In Table 7 the quantitative aspects of the metabolism of benzene and the halogenobenzenes have been summarized. Although all the figures given in this table are not strictly comparable because the sulphur and oxygen conjugations of the chloro-, bromo- and iodo-benzenes are for lower doses than for the other metabolites, they show that fluorobenzene is metabolically similar to benzene rather than to the halogenobenzenes. The main difference between benzene and fluorobenzene on the one hand, and chloro-, bromo- and iodo-benzene on the other, is in the extent of mercapturic acid excretion which is very low for the first two compounds even when the amount excreted unchanged is allowed for, but accounts for nearly 25 % of the last three compounds. The reason for this will not be clear until

Table 6. *Boiling points, vapour pressures and the elimination by rabbits of halogenobenzenes*

Compound	B.p.	Vapour pressure at 30°* (mm.)	Mol.wt.	Percentage of dose eliminated by rabbits in expired air
Benzene	80	117·5	78	39
Fluorobenzene	85	96·6	96	44
Chlorobenzene	132	15·5	112·5	27
Bromobenzene	155–6	5·7	157	6·3
Iodobenzene	189	1·5	204	3·3

* From Young (1910).

Table 7. *Summary of the major metabolites of benzene and the halogenobenzenes*

(Values are expressed as % of dose.)

Compound	Glucuronide	Ethereal sulphate	Mercapturic acid	Unchanged	Total
Benzene	11	25	1	39*	76
Fluorobenzene	10	21	1·6	44	77
Chlorobenzene	25†	27†	20†	27	99
Bromobenzene	40†	37†	21†	6	104
Iodobenzene	31†	30†	23†	3	87

* Parke & Williams (1950). † Values taken from Spencer & Williams (1950); doses low.

some explanation of the high conjugation of chloro-, bromo- and iodo-benzene with cysteine has been found. Experiments directed towards finding this explanation are now in progress.

Since fluorobenzene behaves metabolically like benzene it might be expected to give rise to a muconic acid as does benzene. Theoretically fluorobenzene could give rise to an α- or a β-monofluoromuconic acid (COOH.CH:CH.CH:CF.COOH and COOH.CH:CH.CF:CH.COOH). Both these acids on β-oxidation should yield fluoroacetic acid which is a highly toxic substance (McCombie & Saunders, 1946; Saunders, 1947; Buffa & Peters, 1949). Fluorobenzene, however, is relatively non-toxic, and on these grounds it seems unlikely that it gives rise to fluoroacetic acid *in vivo*. So far we have found no evidence for the formation of a fluoromuconic acid from fluorobenzene in rabbits. We have shown that 0·6 % of benzene is converted to muconic acid in the rabbit (Parke & Williams, 1951). If fluorobenzene were converted to fluoromuconic acid to the same extent and fluoromuconic acid were completely metabolized to fluoroacetic acid, then a dose of 0·5 g. of fluorobenzene/kg. would give rise to 2·3 mg. of fluoroacetic acid/kg. On feeding muconic acid, however, to rabbits, only 40 % is metabolized (Parke and Williams, unpublished observations). It is possible, therefore, that 0·5 g. of fluorobenzene/kg. could give rise to 0·92 mg. of fluoroacetic acid/kg.; this is nearly four times the $L.D._{50}$ of fluoroacetic acid which is 0·25 mg./kg. (Saunders, 1947). However, until fluoromuconic acid or some other fluorine-containing open-chain compound has been isolated from fluorobenzene urine further speculation regarding the formation of fluoroacetic acid seems unprofitable.

SUMMARY

1. A study has been made of the elimination, in the expired air, of unchanged fluoro-, chloro-, bromo- and iodo-benzene after oral administration of these substances to rabbits.

2. A spectrophotometric method for the determination of these halogenobenzenes in expired air has been described. The ultraviolet absorption spectra of these compounds in ethanol are recorded and discussed.

3. Of doses of 0·5 and 1 g. of fluorobenzene/kg., 44 and 64 % respectively are eliminated unchanged in the expired air. At a dose level of 0·5 g./kg. 27 % of chloro-, 6 % of bromo- and 3 % of iodo-benzene is excreted unchanged in the expired air. These results are discussed.

4. The elimination of fluorobenzene in the urine as ethereal sulphates, glucuronides and mercapturic acid has been studied quantitatively and the results suggest that it behaves metabolically like benzene rather than the other halogenobenzenes. Whereas chloro-, bromo- and iodo-benzenes yield large amounts (about 25 %) of mercapturic acid in the urine, benzene and fluorobenzene yield only small amounts (about 1–2 %).

5. Fluorobenzene in single doses up to 1 g./kg. is not highly toxic to rabbits.

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Observations on the Application of Fluorodinitrobenzene to the Quantitative Analysis of Proteins

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The serial micro-estimation of amino-acids has been effected in two main ways: by partition chromatography of the acetamino-acids (Martin & Synge, 1941), and by separation of amino-acids by partition chromatography on starch (Moore & Stein, 1948). A few amino-acids have also been estimated under special circumstances as their dinitrophenyl (DNP) derivatives, but the available methods of separating these compounds are not suitable for a routine procedure for micro-analysis (Sanger, 1945; Porter & Sanger, 1948; Blackburn, 1949). The quantitative nature of the reaction between fluorodinitrobenzene (FDNB) and amino-acids is very attractive, however, and the discovery of a new method of separating the DNP derivatives has led to a reinvestigation of their use in analysis.

The resolution of mixtures of DNP-amino-acids is usually achieved by the serial use of partition chromatographic columns as suggested by Sanger (1945) and by Blackburn (1949). Sanger (1945) has also reported trials with adsorption chromatography, but with the systems he used had only indifferent success. It has now been found that adsorption on kieselguhr permits the discrimination of all the commonly occurring ether-soluble DNP-amino-acids on a single column. This technique has been used as the basis of the analytical procedure which is the subject of this communication.

The absorption method has been applied to several proteins with promising results, but those chosen to illustrate its use here are three for which the composition is known with a considerable degree of probability, namely salmine, insulin and lysozyme. The values obtained have not, however, been fully checked by means of *ad hoc* control mixtures.

EXPERIMENTAL

It will be convenient to describe first of all the coupling of FDNB with a protein hydrolysate, for which certain special pieces of apparatus have been designed.

It is desirable, for reasons to be explained in a following paragraph, to oxidize the protein with 100 vol. hydrogen peroxide in order to convert the cystine to cysteic acid. But if even traces of peroxide are allowed to come into contact with FDNB, a large amount of brown substance is formed which will mask the subsequent chromatographic separation. The oxidation has therefore been done in formic acid according to Sanger (1947), before the protein is hydrolysed. By careful evaporation *in vacuo* most of the hydrogen peroxide can be removed, any that remains being decomposed during the hydrolysis with HCl. The minute amounts of chlorine that are formed are apparently without noticeable effect on the amino-acids.

Hydrolysis. An amount of oxidized protein equivalent to about 0·5 mg. of protein N was boiled with 6 N-HCl on an oil bath at about 130° for 18 hr. The resulting solution was then filtered if necessary and evaporated to dryness *in vacuo*. The amino-acids were dissolved in 2 or 3 ml. of water, and the total N of this solution estimated by micro-Kjeldahl. Based on this value a sample containing approximately 250 μg. N was taken for conversion to the DNP derivatives.

Preparation of the DNP derivatives. The coupling reaction was performed in a reaction tube which was made from a standard *B*14 socket and had a total volume of about 10 ml. This is shown attached to the lower end of the extractor in Fig. 1. Into this tube was pipetted the mixture of amino-acids dissolved in about 1 ml. of water. Residual HCl was neutralized by the careful addition of a slight excess of sodium bicarbonate and followed by about 2 ml. of ethanol. The amino-acids were now in solution in 66% ethanol, not exceeding 5 ml. in volume, and to this were added two drops (0·05 ml.) of FDNB. The reagent used in these experiments was supplied by L. Light and Co. Ltd. and no further purification has been found necessary.

After the tube had been closed with a carefully greased stopper, it was shaken vigorously for a time dependent on the amount of glutamic or aspartic acid present. In several trials with proteins like gliadin, which are rich in glutamic acid, the recoveries of this acid were low, but if the time of reaction with FDNB was increased from 2 to 5 hr. a quantitative recovery resulted. This effect was not found when solutions of pure glutamic acid were used, probably because the quantities taken were too small, but it appears that in a protein hydrolysate the dicarboxylic amino-acids coupled more slowly with FDNB than was expected. Accordingly, if either of the dicarboxylic acids was found to contribute 10% or more of the total protein N, the reaction was allowed to proceed for at least 4 hr. When the amino-N contribution was less than 10%, a coupling time of 2 hr. was sufficient.

It has been found essential during the course of this work to protect the DNP-amino-acids from light at all stages of their preparation and separation. This particularly applies to solutions in alkali and in $CHCl_3$, as has been observed by Blackburn (1949). While being shaken with FDNB, therefore, the reaction tube was enclosed in a short length of the corrugated rubber tubing used on gas-masks.

Purification of the DNP derivatives. When the reaction was completed, the mixture contained excess FDNB, dinitroaniline, dinitrophenol and DNP-amino-acids. The removal of

the first two of these has been done in the extractor (shown in Fig. 1) which has a *B* 14 socket at the lower end, on to which the reaction tube was fitted. The extracting solvent could be boiled in a flask attached to the side arm and was returned down the central funnel by a reflux condenser at the top.

After the reaction tube had been opened, the neck was carefully washed down with a few ml. of distilled water. The tube was then plugged on to the bottom of the extractor and the stopper very carefully washed into the top. Sufficient 0·2N-NaOH was added to make the volume in the extractor about 35 ml. and a brisk stream of ether passed through the mixture until no more yellow material was removed. This should not take more than 10 min.

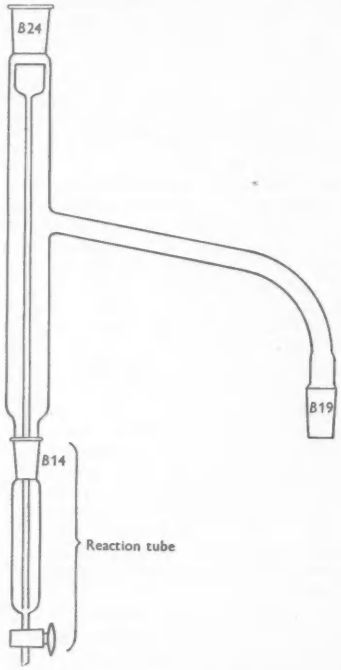

Fig. 1. The special extractor, showing the reaction tube in position.

The apparatus was then tilted, to swill some ether down the side arm and wash away any residue resulting from evaporation of the solvent. The boiling flask was next replaced by one of the type shown in Fig. 2, the ground-glass joint being sealed, as in the first case, with a drop of water. One ml. of 6N-HCl was afterwards pipetted down the central funnel of the extractor and the DNP-amino-acids removed in a brisk stream of ether. When the extraction was nearly complete a small chip of marble was dropped down the funnel, where it fell into the narrow tube above the tap and by its effervescence ensured that this dead space was stirred up. During the whole of the operations in this apparatus the contents were protected from the light by a shield which is not shown in the figure.

When the extraction had been completed, the side arm was again swilled down with ether and the boiling flask removed. The solvents were evaporated over a steam bath to leave a film of yellow DNP compounds round the inner surface of the flask. This mixture contained too much dinitrophenol to be separated on a column, so the amount was reduced by vacuum sublimation in the following way. The cold-finger condenser, which fits the flask containing the DNP-amino-acids as shown in Fig. 2, was loaded with crushed dry ice and the assembly evacuated on an oil pump. When the flask was warmed in a steam bath the dinitrophenol sublimed on to the cold finger. The residue was then redissolved in ether, which was evaporated to leave a new film round the flask, a second sublimation usually being sufficient to reduce the amount of dinitrophenol to manageable proportions.

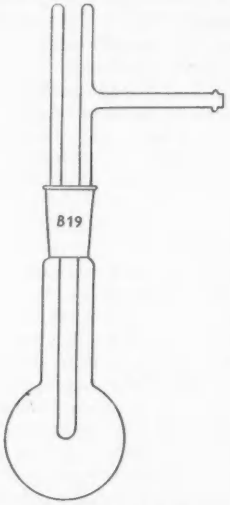

Fig. 2. The cold-finger condenser on to which the dinitrophenol is sublimed.

In the processing of the DNP-amino-acids not extracted by ether, the aqueous phase was drawn into a long-necked flask through the tap at the bottom of the reaction tube. After concentration to small bulk the solution was transferred to a small volumetric flask and this process repeated with the washings from the extractor. Like the ether-soluble compounds, this solution was stored in a refrigerator in the dark.

Chromatographic technique. The kieselguhr used was the 'acid washed' preparation of British Drug Houses Ltd., which was 'activated' before use by shaking up in a large stoppered bottle, inside the stopper of which was fixed a small cloth bag containing a lump of $(NH_4)_2CO_3$. The pH of an aqueous extract of this prepared adsorbent was between 6 and 7.

The series of solvents used with the kieselguhr was of mixtures of methyl ethyl ketone (MEK) and $CHCl_3$, saturated with water. The presence of the water resulted in sharper bands than could be obtained without it, but as the system would also work under completely anhydrous

conditions, the mechanism is presumably one of adsorption rather than partition. The solvents were distilled before use following the observation that old samples of MEK contained a substance reducible by titanous chloride.

For brevity the solvents will be referred to subsequently as 45% MEK, 75% MEK, etc.; this being understood to mean 45% MEK-55% $CHCl_3$ by volume, etc., each mixture being saturated with water at room temperature.

The preparation of columns of kieselguhr was rather more difficult than, for example, in the case of silica gel. Owing to the fact that kieselguhr will not sink very readily in MEK-$CHCl_3$ mixtures, it was necessary to press the solid down with a ramrod and a certain degree of skill had to be acquired before consistently good columns could be made. In a column 1·5 cm. in diameter, it was found that 6·5 g. of dry kieselguhr compressed in 45% MEK-$CHCl_3$ to a height of 12 cm. gave good separations provided great care was taken to ensure that the top of the solid was firm and flat.

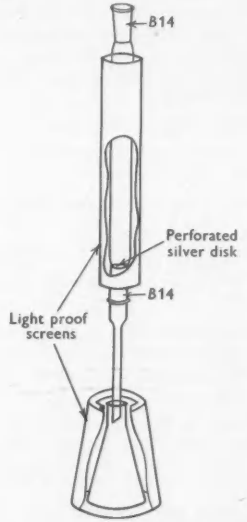

Fig. 3. The chromatographic column, showing the arrangement of light shields.

A column of the kind shown in Fig. 3 has been used in the majority of these experiments. It was made by joining two *B*14 sockets end to end giving a tube about 1·5 cm. in diameter and approximately 24 cm. long. The upper end of the cone leading to the delivery tube was a convenient ledge on which to rest a perforated silver disk covered with a circle of glass cloth. Into the socket at the upper end of the column was fitted a reservoir for solvent, to which a slight pressure of air could be applied.

The solid ether-soluble DNP derivatives were next dissolved by pipetting into the long-necked flask containing them an appropriate volume of 45% MEK. A portion of this solution was then transferred to the column and allowed to sink in. After the sides of the tube had been washed down two or three times with about 1 ml. of solvent, and each allowed to sink in, the reservoir was filled with 45% MEK. A light-proof shield of gas-mask tubing was put round the column, and sufficient pressure applied to cause the delivery of not more than one drop of solvent/sec.

The separate bands of DNP-amino-acids were collected individually, as they reached the bottom of the column, in 50 ml. conical flasks which were stored in a refrigerator. The solvent was evaporated when necessary by directing a stream of air into the flask while it was warmed in a water bath. The solid DNP compounds could then be stored in the cold and dark for several days without apparent deterioration.

The amount of material which was applied to the column depended on the number of components present and their relative abundance. Under most circumstances, however, a column of the size quoted above would carry from 30 to 40 μg. of protein N satisfactorily.

Separation of the water-soluble components. The aqueous phase contained only DNP-cysteic acid and DNP-arginine, which were mixed with various inorganic salts. The latter interfered considerably with the usual chromatograms and were removed in the following way. A portion of the solution was transferred to a porcelain dish and evaporated to dryness on a steam bath. The residue was then washed repeatedly with small quantities of a 10% solution of 6N-HCl in methyl ethyl ketone. This did not dissolve the salts but extracted the DNP derivatives, from which the solvent was afterwards removed by evaporation.

A column of kieselguhr prepared in 75% MEK was used for separating these two amino-acid derivatives which were applied by dissolving the solid in the smallest amount of pure MEK saturated with water. Subsequent development with 75% MEK caused the DNP-arginine to run very fast, while the DNP-cysteic acid remained at the top of the column. After the arginine derivative had been collected, the DNP-cysteic acid could be made to move slowly in 100% MEK.

These two derivatives were then estimated in the same way as the others.

Determination of the DNP derivatives. The procedure was that to be found in most text-books on quantitative analysis, and involved the back titration of an excess of titanous chloride with standard ferric alum solution. The concentration of ferric alum in these experiments was arranged so that 1 ml. was equivalent to about 7·5 μg. nitro-N. Commercial titanous chloride was diluted to make 500 ml. batches and standardized frequently during use, since the solution underwent fairly rapid decomposition.

5 ml. of 0·5N-HCl and 2 ml. of 10% potassium thiocyanate were run into each 50 ml. flask and raised to boiling point under a stream of carbon dioxide. After addition of an excess of titanous chloride the solution was boiled for 3–5 min. according to the amount of DNP derivative present. The flask was cooled under running water before titration of the remaining titanous chloride, carbon dioxide being passed the whole time.

RESULTS

Comparative experiments with solutions of known mixtures of DNP-amino-acids indicated that under the optimum conditions all the common derivatives could be separated on one column of kieselguhr. There does seem, however, to be a number of factors governing the resolution obtained which have not been kept under complete control. Thus it has not

always been possible to separate DNP-leucine from DNP-isoleucine or bis-DNP-lysine from bis-DNP-tyrosine. Some evidence was found that the water content of the developing solvent may be involved. Although difficulty was sometimes experienced with other bands also, this was usually interpretational, being especially noticeable when some bands were faint. If one band was not seen, it was easy to identify some of the remainder wrongly.

The order in which the ether-extracted DNP-amino-acids were eluted from the column is shown in Table 1.

Table 1. *Order of elution of the DNP-amino-acids from kieselguhr, the fastest moving bands being those at the bottom of the table*

(Percentage MEK indicates volume proportion of methyl ethyl ketone in methyl ethyl ketone-chloroform mixtures.)

DNP-amino-acid	Eluting solvent
Bis-DNP-cysteine Aspartic acid Glutamic acid	100% MEK
Serine Bis-DNP-histidine	75% MEK
Methionine sulphone Threonine Glycine Alanine Proline Bis-DNP-tyrosine Bis-DNP-lysine Cystine Valine Leucine Isoleucine Phenylalanine Dinitrophenol	30% MEK followed by 45% MEK

It was necessary at some stage to develop the chromatogram with 30% MEK for a short while, in order to resolve DNP-threonine and DNP-glycine which ran at the same speed in 45% MEK. Fortunately 30 and 45% MEK could be mixed without causing water to be precipitated and could be interchanged on the column without ill effect; thus, the separation of DNP-threonine and DNP-glycine could be made at any convenient time.

It will be seen that DNP-cystine ran close to valine in a part of the chromatogram which was already overcrowded. For this reason the cystine of protein hydrolysates was oxidized to cysteic acid which was not extracted into ether.

Under the usual conditions of extraction, bis-DNP-histidine is also looked for in the aqueous phase (Sanger, 1945). By the technique described above, however, it was obtained in the ether extract and when run on kieselguhr was found to develop three bands. Of these the upper was the strongest and the middle one very weak. No explanation of this behaviour has so far been suggested. The DNP derivative of methionine was also found to give three bands which have been tentatively identified as corresponding to the amino-acid, the sulphone and the sulphoxide. Oxidation of the mixture with hydrogen peroxide resulted in one band increasing in strength at the expense of the other two, which disappeared. It was convenient to identify methionine as the DNP derivative of its sulphone obtained in this way as confusion with the histidine bands was thereby lessened. However, as threonine, methionine and histidine often occur in proteins in comparatively small amounts, the bands corresponding to these amino-acids were sometimes difficult to distinguish.

As a preliminary test of the method, known amounts of the amino-acids glutamic acid, glycine and valine were separately estimated. For the glutamic acid a solution was made containing about 1·0 mg. of amino-acid N/ml. and 10 μl. portions spotted on to a piece of filter paper with a calibrated syringe. The paper was then folded concertina-wise and put into the reaction tube where it was allowed to react with FDNB as described above. A separate experiment showed that no interfering substances were derived from the paper. The estimates averaged from 98 to 99% of the 9·3 μg. of glutamic acid N taken.

Table 2. *Recovery of some amino-acids submitted to the analytical procedure*

Amino-acid	N taken (mg.)	N recovered (mg.)	Recovery (%)	Range (%)
Glutamic acid	0·0093	0·0092	99	±1
Glycine	0·518	0·515	99·4	±0·5
Valine	0·515	0·513	99·7	±0·5

For the glycine and valine 0·5 ml. of a solution containing about 10 mg. N/ml. was treated with FDNB and a portion of the DNP derivative run on the column. Table 2 summarizes the results. The nitrogen in each solution was determined by the micro-Kjeldahl method.

These results were thought sufficiently encouraging to warrant a more stringent trial under the conditions of protein analysis. The proteins examined were a sample of salmine which was the gift of Dr G. R. Tristram, crystalline insulin obtained from Boots Pure Drug Co. Ltd. and crystalline lysozyme marketed by Armour and Co. In each case hydrolysates were prepared as described in the experimental section, and the figures quoted below represent an average of two or three separate analyses on as many different hydrolysates. In all cases the values are the amino-acid N as a percentage of the protein N.

Table 3. *Amino-acids of salmine*

(Amino-acid N expressed as % of protein N.)

Amino-acid	Mills	Tristram (1947)	Block & Bolling (1945)
Arginine	90·6	89·0	92·4
Isoleucine } Valine }	1·85	1·76	2·09
Proline	2·16	2·29	3·11
Alanine	0·59	0·56	1·83
Glycine	1·97	1·78	—
Serine	2·70	3·94	3·02
Total	99·87	99·33	102·45

The estimates obtained for salmine are compared in Table 3 with those given by Tristram (1947) and Block & Bolling (1945). The DNP derivatives of valine and isoleucine in this early experiment were not resolved sufficiently well to permit their individual determination, and they were accordingly estimated together. With the exception of serine, the results agree fairly closely with those of Tristram. For serine a much lower recovery was obtained, being in better agreement with that found by Block & Bolling.

Table 4. *Amino-acids of insulin*

(Amino-acid N expressed as % of protein N.)

Amino-acid	Mills	Chibnall (1946)	Brand (1946)	Fromageot (1950)
Phenylalanine	4·4	4·4	4·2	4·6
Isoleucine	2·1	1·7	1·9	1·1
Leucine	8·1	9·3	8·9	10·2
Valine	6·8	5·8	6·6	5·8
Lysine	3·4	3·0	3·1	3·4
Tyrosine	5·6	6·0	5·9	6·1
Proline	1·7	2·0	2·2	1·6
Alanine	4·4	4·4	—	4·7
Glycine	5·5	5·2	5·4	5·5
Threonine	2·0	1·6	2·3	1·8
Histidine	9·0	8·5	9·0	8·8
Serine	4·7	4·5	4·8	4·5
Glutamic acid	11·1	11·4	12·0	12·0
Aspartic acid	4·5	3·8	4·5	3·8
Arginine	6·1	6·2	7·0	6·0
Cystine	8·0	9·4	8·0	9·5
Total	87·4	87·2	85·8	89·4

Very accurate analyses of crystalline insulin have been published by Chibnall (1946), by Brand (1946) and by Fromageot (1950). This protein was consequently of special use as a test substance. The values quoted in Table 4 are in substantial agreement with those previously published, although in some cases there is greater similarity to the results of Brand than to those of Chibnall or Fromageot. This is noticeable, for example, in the amounts of histidine, aspartic acid and cystine. It will be noticed also that the total leucine N is rather lower than the values quoted in the three other analyses, while the value for valine is high, being comparable with that found by Brand. It is thought that the leucine and valine bands were adequately resolved in these experiments, and consequently that the possibility of their intermixture is unlikely. In one experiment a faint, unidentified band was observed close behind the valine band. This may be due to an interfering substance which was not usually separated from the valine derivative and which spuriously raised the recovery of valine. A similar observation was made by Tristram (1946) during his analyses of insulin via the acetamino-acids.

Table 5. *Amino-acids of lysozyme*

(Amino-acid N expressed as % of protein N.)

Amino-acid	Mills	Fromageot (1950)
Phenylalanine	1·3	1·1
Isoleucine	2·8	3·6
Leucine	3·7	4·0
Valine	4·2	4·0
Lysine	6·3	6·2
Tyrosine	1·4	1·6
Proline	1·1	0·9
Alanine	5·1	5·2
Glycine	4·8	5·6
Methionine	1·2	1·2
Threonine	2·9	3·3
Histidine	1·9	1·5
Serine	4·5	5·1
Glutamic acid	2·2	1·7
Aspartic acid	6·8	6·7
Arginine	25·8	25·6
Cystine	5·8	5·0
Total	81·8	82·3

As for insulin, the analyses of crystalline lysozyme show a fair measure of agreement with the results published by Fromageot (1950), the most substantial differences being in the values for isoleucine, glycine, serine and cystine. In a more recent paper, however, Fromageot, Acher & Jutisz (1950) have given revised results for some amino-acids, which do not conform so well with those in Table 5. It would appear that for these estimations Armour lysozyme which had been twice recrystallized was used. This may explain the source of the different results, since the protein used in the present study was not recrystallized, and especially as Stein has recently resolved lysozyme into two components (Tallan & Stein, 1951).

The most notable modification in the revised figures is to the values for arginine and aspartic acid, the former being diminished to 22·8 % and the latter increased to 9·3 % of the protein N. The value for lysine has also been slightly increased to 7·6 %, while the values for isoleucine, leucine and valine have been readjusted to 3·0, 4·8 and 3·0 % respectively. The results of the DNP method of analysis do not agree with these modifications except in the case of isoleucine. It is worth noting, however, that the total N contributed by isoleucine, leucine and valine is almost the same in each case.

DISCUSSION

The application of a proposed serial method of determining amino-acids will necessarily depend ultimately on the ability of the chromatographic machinery to resolve the amino-acids to an adequate degree. It would appear from experience so far gained in the use of kieselguhr columns that it is possible to separate all the common DNP-amino-acids on this adsorbent. Occasional unexplained failures have been met, however, usually in the resolution of closely running pairs such as DNP-isoleucine and DNP-leucine. In this connexion it seems that the amount of adsorbent/ml. of column may play a critical part. Thus, improved separations of some fast moving derivatives have been obtained by compressing the lower part of the column more tightly than the upper part.

The greatest difficulty in the use of adsorption columns is usually the tendency of the adsorbate to 'tail' during the run. This has been observed under unfavourable conditions on kieselguhr, but does not usually happen with the selected solvents mentioned above. The presence of water in the organic phase has a marked effect in suppressing 'tailing' and some DNP-amino-acids tend to 'beard' if a solvent containing much water is used to elute them. Thus it seems that clear-cut bands depend to some extent on the composition of the solvent, which can in turn be varied, within limits, to obtain the most satisfactory results.

The original separation procedure of Sanger (1945), Porter & Sanger (1948) and the more recent ones of Blackburn (1949), Perrone (1951) and Bell (1949), while giving quantitative separations of the amino-acids, suffer from the disadvantage that several columns are used. In these systems, therefore, numerous quantitative transfers may be necessary. For this reason the kieselguhr column seems to be useful at least for rapid routine analyses of moderate accuracy.

The precision with which estimations can be made is good for single amino-acids, or for simple mixtures, replicate results agreeing to within about $\pm 0{\cdot}5\,\%$. For protein hydrolysates, for which separation of the components may be more difficult, the results may be duplicated to about $\pm 2{\cdot}5\,\%$ in unsatisfactory cases, although it is frequently much better than this.

With a column of the size used here, quantities of amino-acid N of the order of $1{\cdot}0\,\mu$g. could be determined with reasonable accuracy, but bands corresponding to $0{\cdot}5\,\mu$g. or less were so faint as to be difficult to see. Generally, between 2 and $3\,\mu$g. of amino N proved the most suitable concentration for each band.

Kieselguhr also has a considerable sensitivity towards differences in structure, which makes it very suitable for the separation of DNP-peptides. Considerable use has been made of columns for this purpose, up to twenty DNP-peptides being separated on a single unit. The method is also applicable to DNP-proteins, with the modifications necessary to get the particular protein derivative into solution.

SUMMARY

1. A method has been described whereby the dinitrophenyl derivatives of the commonly occurring amino-acids may be distinguished on a single chromatographic column.

2. The application of this technique to the quantitative estimation of amino-acids has been discussed.

3. Analyses of the proteins salmine, insulin and lysozyme are reported as examples of the use of the method.

It is my privilege to acknowledge the support of Prof. E. C. Dodds, F.R.S., in these investigations, and also the value of the many discussions with my colleagues, Dr D. M. P. Phillips and Dr Joan M. L. Stephen.

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The Reaction between Proteins and Reducing Sugars in the 'Dry' State. Relative Reactivity of the α- and ε-Amino Groups of Insulin

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In a study of the reaction between casein and glucose in freeze-dried systems Lea & Hannan (1950) obtained an indication that the few terminal α-amino groups present in casein possess a reactivity towards glucose of the same order as that of the ε-amino groups of the lysine residues. Gottschalk & Partridge (1950), on the other hand, found that in the reaction between amino-acids and reducing sugars the reactivity of the amino group increases as its distance from the carboxyl group increases. Since this latter observation leads to the expectation that the ε-amino group of lysine will react with aldoses much more rapidly than α-amino groups, it was considered desirable to make a further examination of the reactivity of free amino groups in different amino-acid residues in proteins.

The nature and reactivity of the free amino groups of casein were first re-investigated by the fluorodinitrobenzene (FDNB) method (Sanger, 1945), using paper chromatography (Davis, 1950) and reverse-phase partition chromatography on chlorinated rubber (Partridge & Swain, 1950) for the separation and identification of the 2:4-dinitrophenyl-(DNP-) amino-acids. Terminal α-amino groups of aspartyl, glutamyl, lysyl, phenylalanyl and valyl residues were identified, and these α-groups were found to react at approximately the same rate as the ε-amino groups of the lysine side chains. The original observations of Lea & Hannan (1950) were thus confirmed and extended.

The results with casein, however, have not been reported in detail since (*a*) the casein used had been purified from a commercial source and (*b*) the very small proportion of α-amino groups present in casein made a sufficiently accurate determination of their relative activities impossible. For the latter reason, attention was turned towards the reaction of glucose with insulin, a protein in which the proportion of free α-amino groups is exceptionally high, and in which the number, nature and position of all the residues carrying free amino groups have now been established with some certainty (Sanger, 1945; Sanger & Tuppy, 1951).

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EXPERIMENTAL

Determination of optimum relative humidity for the reaction. Crystalline insulin in approximately 3% solution at pH 7 was mixed with 1·5 equiv. of glucose (amino N basis), and part of the solution retained for determination of total N content. The remainder, after dividing equally between a number of glass tubes, was freeze-dried and kept at 37° over solutions of H_2SO_4 which provided a range of constant relative humidities (R.H.). Samples were removed at suitable intervals for determination of amino N by the Van Slyke method, using a reaction time of 30 min. at 20°.

Preparation and standardization of the DNP derivatives of insulin and of insulin-glucose. Two portions of the insulin-glucose mixture (approx. 150 mg. each) were allowed to react at 37° and 65% R.H. for 4 and for 16 days, and immediately treated with FDNB (Sanger, 1945). DNP-insulin (130 mg.) was prepared similarly as a control. The protein content of the DNP derivative was determined in each case by estimating its glutamic acid content by the specific decarboxylase method (Gale, 1945) after hydrolysis with 5·8N-HCl for 24 hr., the glutamic acid content of insulin being taken as 18·60 g./100 g. dry ash-free protein (Chibnall, 1946). This method requires considerably less material than the more usual one based on the determination of amide N (Sanger, 1945).

Chromatographic separation of the DNP-amino-acids. The three DNP proteins (30–55 mg.) were hydrolysed with 10 ml. 5·8N-HCl for 4 hr. and the hydrolysates extracted with ether. DNP-glycine and DNP-phenylalanine were determined in the ether extracts after separation by partition chromatography on silica gel-$CHCl_3$ columns. The silica gel used failed to give a satisfactory separation of the DNP-phenylalanine band from the artifact band, and the latter was therefore removed before running the chromatogram by the following method due to Isherwood & Cruickshank (1951). The ether-soluble DNP-amino-acid fraction was dissolved in 90% (w/v) H_2SO_4 and extracted with benzene to remove dinitrophenol. The H_2SO_4 solution was then diluted with ice water and extracted with benzene containing 10% (v/v) *tert.*-amyl alcohol. The DNP-amino-acids and any dinitroaniline present passed into the benzene-*tert.*-amyl alcohol solution, from which the DNP-amino-acids were extracted with 1% (w/v) $NaHCO_3$ solution. The DNP-glycine and DNP-phenylalanine were recovered from the $NaHCO_3$ solution by acidification and extraction with ether. They were separated from each other by partition chromatography on a silica gel-$CHCl_3$ column (Sanger, 1945) and estimated spectrophotometrically in 1% $NaHCO_3$ solution.

The HCl-soluble residue from ether extraction of the original hydrolysate was evaporated to dryness in a rotary

film vacuum evaporator (Lea, Hannan & Rhodes, 1951) and refluxed with 10 ml. 5·8N-HCl for a further period of 8 hr. ϵ-DNP-lysine was estimated spectrophotometrically in N-HCl solution after separation on a silica gel-66% methyl ethyl ketone-ether column (Sanger, 1945).

Recovery experiments. Recovery experiments were carried out, after acid hydrolysis, on a mixture containing DNP-glycine, DNP-phenylalanine, ϵ-DNP-lysine and protein in the proportions present in DNP-insulin. Both casein and a casein-glucose complex were used as proteins, the latter being included to test whether the larger amount of humin formed during the hydrolysis of protein-sugar complexes affects the recovery of DNP-amino-acids.

The values obtained in the presence of protein were very close to those of Sanger (1945), indicating that the extra steps involved in removal of the artifacts do not result in any appreciable loss of the DNP-amino-acids. The recoveries, moreover, were not significantly affected by hydrolysis with the protein-sugar complex in place of protein (Table 1).

Table 1. *Recovery of DNP-glycine, DNP-phenylalanine and ϵ-DNP-lysine after hydrolysis with protein or with a protein-sugar complex*

	Hydrolysis time (hr.)	Recovery (%) With casein	Recovery (%) With casein-glucose
DNP-Glycine	4	53·8, 52·6	51·9
DNP-Phenylalanine	4	85·3, 88·7	83·3
ϵ-DNP-Lysine	12	88·4, 88·2	92·4

RESULTS

Influence of activity of water on the amino group-glucose reaction. The effect of storage in equilibrium with atmospheres at 15–85% R.H. on the rate of disappearance of the amino groups of insulin, as determined by the Van Slyke method, is shown in Fig. 1. The proportion of the total amino groups which had reacted with glucose after a given period at 37° was found to increase with increasing humidity to a maximum at about 70% R.H., and then to decrease again at higher humidities. The browning of the reaction mixture which accompanied the loss of amino groups, on the other hand, did not show any evidence of an optimum humidity, but increased progressively with humidity throughout the range covered.

Reaction of insulin with glucose at 65% R.H. Fig. 2 shows the course of the reaction, as followed by the loss of Van Slyke amino N, of insulin with glucose (1·5 equiv.) at pH 7 and 65% R.H. during 32 days at 37°. Approximately one-third and two-thirds of the total amino groups can be seen to have reacted after 4 and 16 days, respectively. At 4 days no browning of the stored material was detectable, but at 16 days the reaction mixture was perceptibly brown and at 32 days more noticeably so.

On the basis of these results larger samples of insulin were allowed to react with glucose under similar conditions for 4 and 16 days for subsequent determination by the FDNB method of the nature and quantity of the free amino groups remaining unreacted.

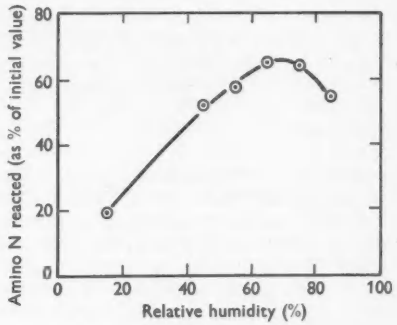

Fig. 1. Influence of humidity on the rate of reaction of insulin with glucose. (Insulin reacted with 1·5 equivalents of glucose/amino group for 16 days at 37°.)

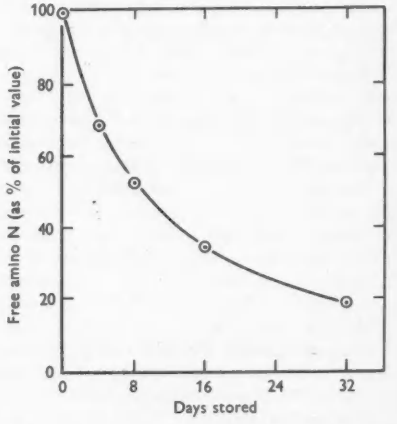

Fig. 2. Loss of free amino N in insulin stored with glucose at 65% R.H. and 37°.

Rates of reaction with glucose of the three types of amino group present in insulin. The results of examination by the FDNB method of insulin and of the 4- and 16-day insulin-glucose reaction products are presented in Table 2 and Fig. 3. The numbers of free amino groups of the three types found in insulin are only approximately equal to the theoretical value of 2·0 each, but the figures are quite close to those previously obtained by Sanger (1945).

The reactivity towards glucose of the three types of amino group obviously varies very considerably, those of phenylalanine being most and those of lysine

Table 2. *Reaction of the α- and ε-amino groups of insulin with glucose at 37° and 65 % R.H.*

	Free amino groups/submolecule of mol.wt. 12,000	Amino N (mg./g. total N)	
		Calc. from FDNB data	Found by Van Slyke method
Insulin			
Glycyl α-	2·31	23·3*	
Phenylalanyl α-	1·81	13·6	
Lysyl ε-	1·91	14·3	
Total	6·03	51·2	49·4
Insulin-glucose (reacted 4 days)			
Glycyl α-	1·46	14·8*	
Phenylalanyl α-	0·55	4·1	
Lysyl ε-	1·52	11·4	
Total	3·53	30·3	30·2
Insulin-glucose (reacted 16 days)			
Glycyl α-	0·77	7·8*	
Phenylalanyl α-	0·36	2·7	
Lysyl ε-	0·86	6·5	
Total	1·99	17·0	16·9

* Calculated on the assumption that glycine gives values 135 % of theoretical in the Van Slyke reaction in a 30 min. reaction period at 20°.

least reactive. In the 4-day reaction product, for example, the free α-amino groups attached to the terminal phenylalanyl residues had reacted approximately twice as extensively as the α-amino groups attached to the terminal glycyl residues, and about 3·5 times as extensively as the ε-amino groups of the lysyl residues. After 16 days the differences between the various groups were becoming less marked as the phenylalanine reaction slowed down on approaching completion (cf. Fig. 2).

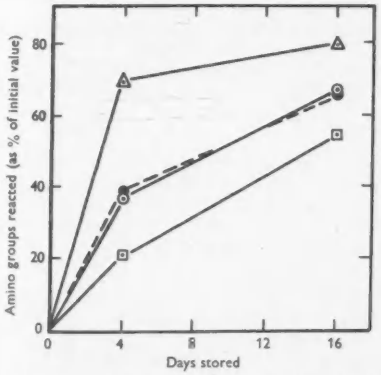

Fig. 3. Reaction of the amino groups of insulin with glucose at 37° and 65% R.H. ⊙—⊙, glycyl α-amino groups (terminal); △—△, phenylalanyl α-amino groups (terminal); ⊡—⊡, lysyl ε-amino groups; ●- - - - -●, total amino N (Van Slyke).

Excellent agreement was obtained between the values for the total amino N content of the insulin-glucose products as determined by the Van Slyke method and as calculated from the FDNB data on the assumption that glycine gives values equal to 135 % of theoretical in a 30 min. reaction period in the Van Slyke determination (cf. Sanger, 1945).

DISCUSSION

The existence of an optimum R.H. for reaction of the amino groups appears to be a characteristic of the protein-sugar reaction in the 'dry' state. Insulin resembles casein (Lea & Hannan, 1949) in showing maximum reactivity of the amino groups at about 70 % R.H., in contrast to the more complex dried blood-plasma system where maximum reactivity is at about 55 % R.H. (Lea, Hannan & Greaves, 1950).

The results obtained from the reaction of the α- and ε-amino groups of casein, and particularly of insulin with glucose are quite different from what might be expected from Gottschalk & Partridge's (1950) observations on the reactivity of simple amino-acids with glucose. It is not unreasonable to suppose, however, that the influence of a neighbouring free-carboxyl group will not be the same as that of a carbonyl group in a peptide linkage; the apparent dissociation constant of the amino group of an amino-acid is known to be altered considerably when the amino-acid becomes part of a peptide.

In proteins, moreover, steric factors might be expected to influence the relative ease with which the rather bulky glucose molecule can approach and react with the amino groups. In the case of insulin it is perhaps not without significance that the most reactive (phenylalanyl) α-amino groups are situated at the ends of the longer '*B*' chains which are

probably on the outside of the protein molecule, and the glycyl α-amino groups are at the ends of the shorter '*A*' chains (Sanger, 1948) and perhaps therefore less accessible, while the least reactive ε-amino groups of the lysyl side chains are now known to be located, one in each '*B*' chain, adjacent to the alanine residue which provides the terminal carboxyl group of these chains (Sanger & Tuppy, 1951).

It is hoped to extend these investigations to other proteins and peptides with the object of obtaining some insight into the factors influencing the reactivity of amino groups towards reducing sugars.

SUMMARY

1. The reaction at 37° between insulin and glucose freeze-dried together from solution at pH 7 has been investigated.

2. The rate of reaction has been shown to increase with increasing activity of water to a maximum at a water content in equilibrium with a relative humidity of approximately 70 %, and thereafter to decrease again with further increase in water content.

3. The reactivities towards glucose of the α-amino groups attached to the terminal phenylalanyl and glycyl residues, and of the ε-amino groups attached to the lysyl residues of insulin have been determined by the fluorodinitrobenzene method. After 4 days at 65 % relative humidity, 70 % of the phenylalanyl amino groups, 37 % of the glycyl amino groups and only 21 % of the lysyl amino groups had reacted. After 16 days the corresponding figures were 80, 66 and 55 %.

The authors wish to express their thanks to Dr R. S. Hannan for helpful discussions on the fluorodinitrobenzene method, to Dr E. F. Gale for glutamic acid decarboxylase, and to Dr F. A. Isherwood for the method used for the removal of interfering substances from hydrolysates of 2:4-dinitrophenyl proteins.

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A Micromethod for the Serial Determination of Organically Bound Halogen

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The main methods for the determination of organically bound halogen are: (1) combustion with nitric acid in the presence of silver nitrate and gravimetric determination of silver halide, (2) dry combustion yielding the free halogen which may be determined gravimetrically or volumetrically, (3) fusion with potassium and volumetric determination of the ionized halide. The authors were faced with the problem of having to follow at frequent time intervals the metabolic fate of halogen-containing compounds in micro-organisms. For this purpose methods (1) and (2) were found to be too long and cumbersome, whilst in the potassium-fusion method difficulty was experienced in bringing all of the organic substance into intimate contact with the potassium. The following alternative method was therefore devised allowing the rapid determination of small amounts of organically bound halogen (1·5–15 μmol.) in series.

PRINCIPLE

The halogen-containing compound is subjected to combustion by fuming nitric acid in the presence of silver nitrate.

The precipitate is collected by centrifugation in the combustion tube, dissolved in water by adding a complex-forming substance (aqueous ammonia in the case of chloride and bromide, and potassium cyanide in the case of iodide) and the silver halogen complex reduced (by hydrazine hydrate in the case of chloride and bromide, and zinc powder in the case of iodide) to metallic silver and ionized halogen.

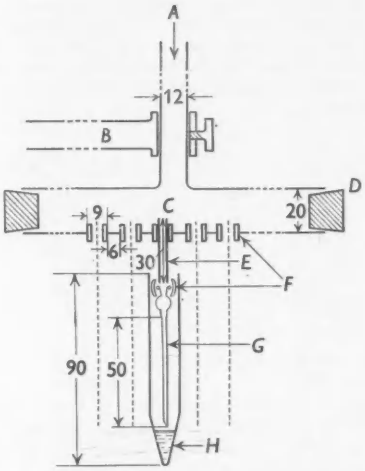

Fig. 1. Evaporation device. *A*, air inlet tube; *B*, support with set screw; *C*, manifold; *D*, cork; *E*, glass tube; *F*, rubber tubing; *G*, hypodermic needle; *H*, combustion tube containing solution to be evaporated. All dimensions in mm.

The metallic silver precipitates and the ionized halogen remains in aqueous solution. (When this work was completed a paper by Iwantscheff (1950) came to the notice of the authors in which a similar principle had been applied to the determination of traces of chloride in selenium.)

The amount of halogen present can be determined by volumetric estimation of the precipitated metallic silver after dissolving in nitric acid or by direct titrimetric determination of the ionized halogen. For the determination of silver and ionized halogen any of the conventional titrimetric methods can be used. The authors have preferred the potentiometric method for the determination of silver, and the diffusion methods of Conway (1947) for the determination of ionized halogen.

EXPERIMENTAL

Reagents (all of analytical grade) for preparation of solutions for titration

Silver nitrate; fuming nitric acid; ammonia, *d*, 0·880; potassium cyanide, 2% (w/v) aqueous solution; hydrazine hydrate, 45% (w/v) aqueous solution; zinc powder; sodium laurylsulphonate, aqueous solution, saturated at room temperature; sodium sulphite; potassium chloride, 0·003 N aqueous solution.

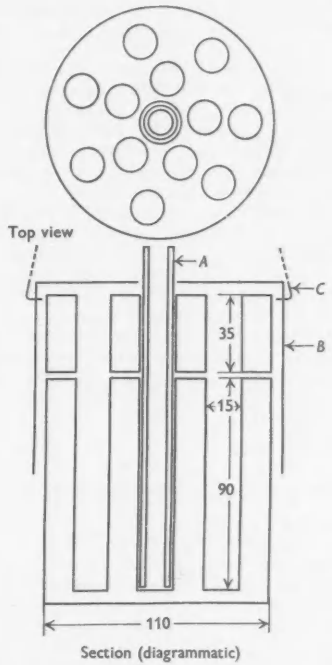

Fig. 2. Micro-Carius furnace. *A*, support for thermometer tube; *B*, tinplate shield; *C*, wire support. All dimensions in mm.

Preparation of the sample to be analysed for combustion

The halogen-containing organic substance to be analysed can be present in aqueous solution or in an organic solvent. The amount to be analysed should be of the order of 1·5–15 μmol. of halogen. A suitable volume of solvent containing the desired amount of halogen was evaporated to dryness in portions of about 1 ml. in combustion tubes which had been carefully cleaned with chromic acid solution. The combustion tubes, length 90 mm., internal diameter 7–8 mm., were made from bomb tubing. These tubes were tapered for about 20 mm. at the bottom to facilitate the collecting of the silver halide precipitates. For the evaporation of the solvent the special arrangement illustrated in Fig. 1 was used. It

consisted of a manifold to which a number of hypodermic needles was fixed in such a way that a fine stream of air could be blown through them. The combustion tubes containing the solution to be evaporated were held in a water bath by means of metal clips and into each tube a hypodermic needle was inserted so that its end was just above the surface of the liquid. The water bath was heated to a temperature just below the boiling point of the solvent and a current of air, as small as possible to avoid splashing, was passed through the needles over the surface of the solution. Under these conditions evaporation was complete in a few minutes.

Combustion and collection of the silver halide precipitates

Solid $AgNO_3$, judged to be about twice the calculated amount required, was added to each tube followed by 0·1 ml. of fuming HNO_3 (in the analysis of iodoacetate, conc. HNO_3 was used because fuming HNO_3 caused rapid decomposition immediately). As much as possible of the substance which adhered to the wall of the tube was washed down with the acid by tilting the tube. The tubes were then sealed with the blowpipe and heated for 6 hr. at 270–300° in a furnace placed in the fume cupboard (for precautions in sealing and opening the tubes see Gattermann & Wieland 1946). This heating time may be considerably reduced. For instance, Niederl, Baum, McCoy & Kuck (1940) find that 300° for 0·5 hr. is sufficient for most halogen compounds. The furnace illustrated in Fig. 2 was made from an Al block which was heated by a Bunsen burner. An adequate safety device was found to be a disused tinplate container which was held above the block by a bucket-handle type suspension. The breakage of the tubes under these conditions was found to be very small.

Collection and reduction of silver halides

Silver chloride and bromide. After cooling and releasing the pressure the tubes were opened just below the shoulder by making a scratch and touching it with a hot glass rod. The HNO_3 was carefully diluted with water (1 ml.), stirred (using a thin drawn-out glass rod) and the tubes were heated on a water bath for 5–10 min. to decompose the silver halide-$AgNO_3$ complex. After the heat treatment, the silver halide precipitate, which should be in a loose powdery form, was centrifuged into the tapered part of the combustion tube, first adding to the contents of the tube a drop of sodium laurylsulphonate solution which serves to prevent particles of the precipitate being held on the surface of the liquid. The supernatant was removed with a Pasteur pipette and the precipitate washed twice on the centrifuge in the same way with water acidified with HNO_3, stirring each time and adding a drop of sodium laurylsulphonate. This technique of collecting precipitates by centrifuging with laurylsulphonate was used throughout this work.

The halide was dissolved by stirring with conc. NH_3 (0·3 ml.). To the solution at room temperature a solution of $N_2H_4.H_2O$ (0·05 ml.) was added. (It is important to carry out the reduction at about 20°; if the solution is warm the Ag is not all precipitated but tends, in part, to form a colloidal solution.) The solution was mixed by rotating the tube between the palms of the hands and after 2 min. standing, water (about 0·5 ml.) and a drop of sodium laurylsulphonate solution were added. The precipitated metallic Ag was centrifuged down. The supernatant was carefully removed and collected in a dwarf tube with a Pasteur pipette. The Ag precipitate was washed once with water (1 ml.), without stirring, collected by centrifuging and the washing added to the first supernatant.

Silver iodide. The AgI precipitate and HNO_3 were diluted with water (1 ml.). To decompose the AgI-$AgNO_3$ complex which is much more stable than that of Cl^- and Br^-, a double-heat treatment was required. The tube was heated on a boiling-water bath until the precipitate was soft enough to be crushed by a thin glass rod with a ball end; the powdered precipitate was then heated for a further 15 min. The tubes were removed from the water bath and to the hot solution was added a little Na_2SO_3 (about 15 mg.) to reduce to I^-, any IO_3^- which might have been formed. The precipitate was collected by centrifuging with sodium laurylsulphonate and the supernatant removed. Water acidified with HNO_3 was added and, after stirring, the precipitate was heated for a further period of 15 min. on the boiling-water bath. The AgI was collected, washed once more with the dilute HNO_3 and finally with water to remove traces of acid.

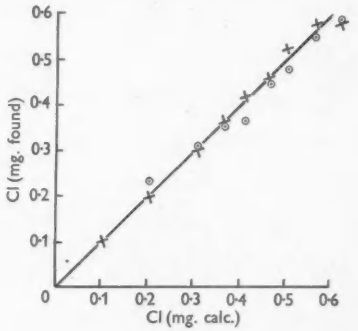

Fig. 3. Chlorine determinations. ×, *o*-Chlorobenzoic acid (argentometric method); ⊙, *o*-Chlorobenzoic acid (Conway method). The line drawn corresponds to that for the theoretical values.

The precipitate was dissolved in the aqueous solution of KCN (0·2 ml.). The reduction of the AgCN complex was carried out by addition of Zn dust (about 30 mg., the large excess being necessary to provide a large surface for the reaction) and stirring for about 1 min. After the reduction, water (1 ml.) and a drop of sodium laurylsulphonate solution were added and the solution centrifuged. The I^--containing supernatant was separated by means of a Pasteur pipette, the precipitate containing metallic Ag and excess Zn was washed once by stirring with water (1 ml.) and after centrifuging, the wash-water was added to the first supernatant.

Titrations

Metallic silver. The Ag precipitates were dissolved in 50% (w/v) HNO_3 by warming (0·2 ml. for the Ag precipitated by reduction of AgCl and AgBr; 0·25 ml. for the precipitate containing Ag and excess Zn, arising from the reduction of AgI). The Ag solutions were then titrated potentiometrically with the standardized KCl solution, using a glass reference

electrode and a continuous-reading electronic voltmeter (see Lykken & Tuemmler, 1942).

Chloride ions. The supernatant solutions from the Ag precipitates and their washings were evaporated to dryness by blowing an air stream over their surfaces as described above. The Cl^--containing residues were transferred to the outer rim of the Conway units in two portions of 0·5 ml. of water and the halogen was determined in the manner described by Conway (1947). A silicone fixative was used as small losses of halogen occurred with the normally used paraffin fixative.

Bromide ions. To the solution containing the Br^- was added 0·05 ml. 10% (w/v) KOH. The solution was evaporated to dryness and the residues were then heated in the oven at 100–110°, for 1 hr. to remove all traces of excess

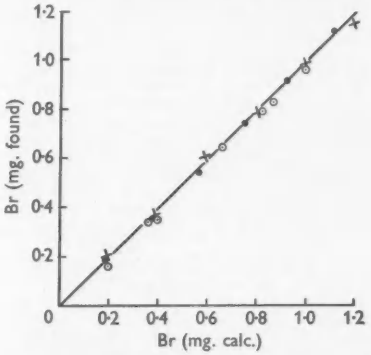

Fig. 4. Bromine determinations. ●, Bromoterephthalic acid (argentometric method); ×, potassium *p*-bromophenylacetate (argentometric method); ⊙, potassium *p*-bromophenylacetate (Conway method). The line drawn corresponds to that for the theoretical values.

$N_2H_4.H_2O$ and then transferred to the Conway units in two portions of 0·5 ml. of water. If the heating was omitted the presence of excess $N_2H_4.H_2O$ in the solution led to N_2 production and the formation of a foam on the surface of the $K_2Cr_2O_7$ solution. This interfered with the diffusion of Br_2 from the outer rim into the inner chamber of the Conway unit. The KOH was added to convert NH_4Br to KBr, since it was found that heating the former salt alone caused a significant loss of Br^-, presumably due to volatilization of the salt. The Conway determinations were carried out in the prescribed manner (Conway, 1947), but using a silicone fixative.

Iodide ions. The tubes containing the I^- solutions were clipped in a water bath placed in the fume cupboard, 0·8 ml. 0·066 M-phosphate buffer (pH 7) was added and the solution was evaporated to dryness by the air-stream method. Thus the CN^- was removed by volatilizing it as HCN. The residues were extracted with two successive 0·5 ml. portions of water and transferred to the outer chamber of the Conway unit. In order to liberate the I_2 0·1 ml. 4·2 N-H_2SO_4 was added, and the determination then carried out as described by Conway (1947), except that a silicone fixative was used. A control experiment showed that under these conditions Br_2 was not liberated from a solution containing Br^-.

RESULTS

The results are given in Figs. 3–5. Slightly lower results were obtained by the Conway method, but this method has the advantage of providing a means of estimating separately chloride, bromide and iodide in mixtures of all three.

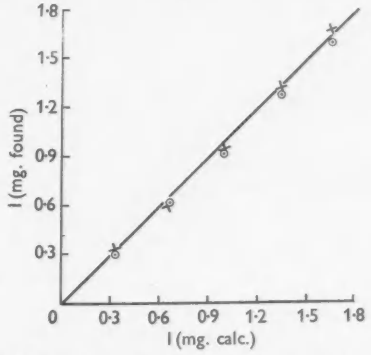

Fig. 5. Iodine determinations. ×, Potassium iodoacetate (argentometric method); ⊙, potassium iodoacetate (Conway method). The line drawn represents the theoretical values.

SUMMARY

1. A titrimetric micromethod for serial determinations of chlorine, bromine and iodine in organic compounds is described.

2. The organically bound halogens are transformed into silver halides by combustion with fuming nitric acid, the silver halides are dissolved by a complex-forming agent (ammonia in the case of chlorides or bromides, and potassium cyanide in the case of iodides) and reduced to metallic silver, which precipitates, and halogen ions, which remain in the supernatant solution.

3. The halogen present can be estimated by determination of the precipitated metallic silver, or by direct titration of the halogen ions in the supernatant solutions using the diffusion methods of Conway.

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